Pharmacological Augmentation of Heavy Ion Cancer Therapy

Vom Fachbereich Biologie der Technischen Universität Darmstadt zur Erlangung des akademischen Grades eines *Doctor rerum naturalium* genehmigte Dissertation von

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Eingereicht am 03.03.2017

Reppingen, Norman: Pharmacological Augmentation of Heavy Ion Cancer Therapy
Jahr der Veröffentlichung der Dissertation auf TUprints: 2018
Tag der mündlichen Prüfung: 06.09.2017

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1 Zusammenfassung

Im Bereich der Krebstherapie gibt es derzeit massive Fortschritte, einschließlich der Felder Strahlentherapie, Immunologie und Pharmakologie. Mechanismen der Therapiemethoden auf der einen Seite und Mechanismen der Therapieresistenz werden immer klarer, was es erlaubt Kombinationstherapien auf die immer noch immensen therapeutischen Herausforderungen zuzuschneiden. Dies betrifft auch die Schwerionentherapie, die lokal sehr effizient ist, deren systemische Wirkung aber noch weitgehend ungeklärt ist. Auf der zellulären Ebene können Änderungen im Phänotyp der Tumorzellen zu einer besseren Erkennbarkeit durch das Immunsystem führen - als Teil von Strahlenantworten and pharmakologischen Mechanismen. In dieser Arbeit betrachten wir Phänotyp-Änderungen auf den murinen Tumor-Zelllinien CT26.WT und 4T1 nach Bestrahlung mit Kohlenstoff - Ionen und Röntgenstrahlung sowie spezifischer Inhibierung von c-Met, um einen Einblick in zugrundeliegende Mechanismen zu erhalten. Danach folgt eine Evaluierung von drei pharmakologischen Wirkstoffen, die förderliche immunmodulierende Effekte gezeigt haben: Chloroquin, CDDO-Me und XL-184 (Cabozantinib). Daher wird hier die Hypothese aufgebracht, daß die immunstimulierenden Eigenschaften, die jeweils in verschiedenen Modellen beschrieben wurden, in der Kombination synergistisch wirken, da die Wirkmechanismen der einzelnen Substanzen verschieden und daher sehr wahrscheinlich nicht redundant sind. In der Kombination mit Kohlenstoffstrahlung und Röntgenstrahlung wurde hierdurch im CT26.WT Zellen ein verstärkter Trend zu apoptotischem Zelltod festgestellt, während insbesondere Kohlenstoffstrahlung alleine eher zu einem nekrotischen (Annexin-V negativem) Zelltod führte. In einem *in vivo* Modell für metastatischen Brustkrebs (4T1) wurde gezeigt, dass XL184 das Tumorwachstum stark verringert. Zusammenfassend könnte die Schwerionentherapie von dem hier vorgestellten Kombinationsansatz profitieren, da die Zelltodmechanismen und damit die Immunogenität möglicherweise unterschiedlich sind.

2 Abstract

Tremendous progress is currently achieved in treating cancer, including the fields of radiotherapy, immunotherapy and pharmacology. Mechanisms of therapy methods on the one hand and treatment resistance mechanisms are increasingly elaborated, allowing to design combined treatment approaches which address the tremendous therapeutic challenges still imposed by many cancerous diseases. This also applies to heavy ion cancer therapy, which is very effective as a single modality treatment, but remains a localized treatment with unclear systemic effects. On the cellular level, changes of phenotype could render tumor cells more visible to the immune system, as part of the response to radiation and by pharmacological mechanisms. In this thesis, we are looking at phenotype changes of murine tumor cell lines CT26.WT, 4T1 following x-ray and carbon ion irradiation as well as specific inhibition of c-Met, MEK and Akt, in order to gain mechanistic insights. Three pharmacological agents are evaluated, which did already show beneficial immune modulatory effects: Chloroquine, CDDO-Me and XL-184 (cabozantinib). Therefore, it is hypothesized here that the immune stimulatory properties seen in different models by others could act synergistically, as the mechanism(s) of activity of each compound are different and therefore most likely non redundant. In combination with carbon ion irradiation and x-ray an increase in apoptotic cell death was found in apoptotic cells, while carbon ion irradiation alone did rather lead to a necrotic (annexin-V negative) cell death. In an *in vivo* model for metastatic breast cancer (4T1) it is shown that XL184 does vastly reduce tumor growth. Conclusively, also heavy ion cancer therapy could potentially draw benefit from the pharmacological approach presented in this thesis, as the cell death mechanism and therefore the immunogenicity are potentially different.

3 Acknowledgements

Of course, this project and the results would not have been possible without the essential contributions from many people. There were decisive activities, adding central parts, and all sorts of kind and helpful contributions for which i am very grateful.

First of all, i am very thankful for **Marco Durante** daring to take up this topic at a time where it was not established at all in the radiology community, and together with Gerhard Thiel and Claudia Fournier Marco Durante was spearheading the PhD - Panel, which collectively enabled to guide the process from thoughts over *in vitro* to *in vivo* experiments quite straightforwardly.

Gerhard Thiel was so kind to examine this thesis.

Harald Kolmar contributed a lot of suggestions in the finalization of this thesis.

Claudia Fournier as the leader of the tissue effects group has despite already great workload even increased her efforts to turn down hurdles and enable things to happen also in this project, which was and is very important. She also bravely took the burden to proof read this thesis.

Corinna and Michael are now herding all of us in a way which i find very helpful, Corinna did contribute with a lot to the project management.

The GSI Biophysics Group as a whole did contribute with important questions and deeds, logistic support, informations and discussions, i have to thank **Aljona Cucu** and **Paul Günther** for being great officemates, **Maria Didonna Schnellbächer** and **Julia Bellartz** for secretarial assistance. **Kateryna Shreder** introduced me to Q-PCR, Aljona and **Eva Thönnes** did show me how to appopriately do western blots which i occasionally halfway managed to reproduce, while **Till Dettmering** introduced me to the FacsCanto II and its ever amusing divaesque software. **Nadine Erbeldinger** and **Aylin Benzer** took care for diligent animal tissue conservation, and **Emilia Choman** was helping a lot to provide an inhabitable workspace (with me messing it all up again). Even though i am not allowed to talk about it, i am also grateful for Petra Hessel introducing me and others to the art of dance.

The TRON Team did carry out all the top notch animal work funded by the GSI under the auspices of **Jutta Petschenka**. **Goran Martic** and **Ines Eckhardt** were involved

in meticulous experiment planning, and the approval of **Ugur Sahin** of course enabled things to take place.

The students **Dominique Tandl and Tobias Schulze** did pioneer the B16-F10 work and undertook a lot of experiments, while **Miriam Kress** was hacking the transcriptomes from the raw read data provided by the TRON crew, which enabled to compare the gene activitiy of the murine tumor cell lines in a straightforward table and did provide a howto manual .

Oliver Kepp & Lorenzo Galluzzi from the group of **Guido Kroemer** (INSERM) provided us with the autophagy related U2OS-LC3-GFP-RFP cells and other cell lines.

Kenneth Rock and the Dana Farber Cancer Institute allowed us to use his DC2.4 cell line, **Veinot Joan** and **Janice Bellelsle** took care sending us these cells, showing a lot of patience for our weird import regulations.

HGSHire and the GSI in general were contributing a lot of funding including the in *vivo work* carried out by the TRON team.

Matthew Ricci, the vice president of researchdiets.com was investing a lot of meticulous efforts to ensure the drugged research diet to match the needs.

Last, but not least, my mother **Erika Schreiber** was providing logistics assistance in the animal project. The extent of humor and patience invested by her in the process which finally led to this thesis is beyond description.

4 Introduction

Cancer[1,2] is caused by increasingly known[3,4] mutations of dividing stem cells[5], spurred by external insults[6] and genetic predispositions[7]. The rapidly expanding knowledge enables improved diagnosis[8,9], identification of new cancer drug targets[4] and tumor specific antigens for immunotherapy[10,11]. Radiotherapy will benefit from inevitable[12,13] individualization of prescription doses and therapy decisions like choosing radiation qualities (e.g. photons versus ions)[14,15] and allow for improved radiation risk estimates[16–18]. Although progress in radiation oncology was driven mainly by physical developments[19] and cell intrinsic insights[20], research on cancerogenic, inflammatory insults, like viral[21,22] or bacterial[23] infections, pointed to the role of normal host cells in cancerogenesis[24–26] and cancer progression[27–29]. With inflammation as a key process[24], the tumor microenvironment emerged as a place reprogramming the host for metastasis[27], suppressing the immune system[30–32], and providing growth factors to tumor cells[33]. Despite tumor heterogeneity[34,35], there is optimism for targeted pharmacological management[36], as the diversity of mutations[4] is converging on few increasingly druggable pathways[5] (see figure 1). With high mutational load, responsiveness to immunotherapy is increasing[37]. But despite recent breakthrough successes e.g. in melanoma[38], immune mediated cure is overall still rather an exception than a rule[39].

However, in cases like juvenile desmoid tumors with very few mutations and no well working drugs at hand[40], cure is readily furnished by protons[41] and carbon ions[42]. In more challenging diseases like pancreatic cancer, carbon ion radiotherapy with concurrent immune stimulating Gemcitabine[43] already furnishes a formerly unseen, but short two year median survival of 43%[44]. Consequently, an integrated pharmacological approach can not be dismissed.[13,45].

Mimicking an immune activating, IFN-I dependent[46] viral insult[10] dependent on STING signaling[46], the success of radiotherapy[47] and some types of chemotherapy[48] are also dependent on the immune system. A key question is how to augment radiotherapy with pharmacological and immunological optimization[13,45]. Clinical trials heading in this direction are ongoing[49], and this is also the main motivation for this thesis.

Scope of the Thesis and Choice of Compounds

From the situation as described above, we can conclude that there is a considerable challenge to appreciate innumerable different patient conditions which are vastly different from one another. The range is from patients with extremely few tumor mutations up to several thousands. It is currently unclear if it will ever be possible to find an effective drug related answer to each of the patient situations. Also, it is currently mostly unclear, which treatment approach, whether pharmacological, immunological or radiological can be most successful in the respective cases. Most crucially, a lot of patients need solutions *now*.

This thesis is directed toward an integrated pharmacological approach, which aims to address as many as possible challenges with as small as possible toxicity. Some of the challenges are cell intrinsic, partly tumor cell intrinsic, and cell intrinsic concerning entire groups of normal host cells which can support tumor growth.

These issues are introduced in the following, divided in an *in vitro* and *in vivo* part. This is followed by a pharmacological profile which is seen as a set of possible answers to the outstanding questions described in these introductions. This is complicated by the fact that each and any treatment, which is thought to act in one part of the host or tumor, inevitably will have effects on other parts and situations. On the one hand, this can result in treatment related complications. But on the other hand, treatment modalities which were thought to be cell intrinsic are *de facto* effective by immune mediated mechanisms. This includes chemotherapy and radiotherapy[50], as well as targeted treatments[51–53], which is now increasingly acknowledged (see Figure 3 to give an example about how this potentially could look like).

The choice of compounds was to a vast degree met by considering pathways relevant for tumor growth and immune suppression, which were integrated in a mindmap (http://radio-immunology.org/Immunity_Irradiation_Cancer_by_Norman_Reppingen.pdf). In the case of CDDO-Me, the Anderson Cancer Center published about 25 research papers exploring the use and properties of this substance (personal communication with Michael Andreeff), encouraging its use to include it in radiotherapy settings.

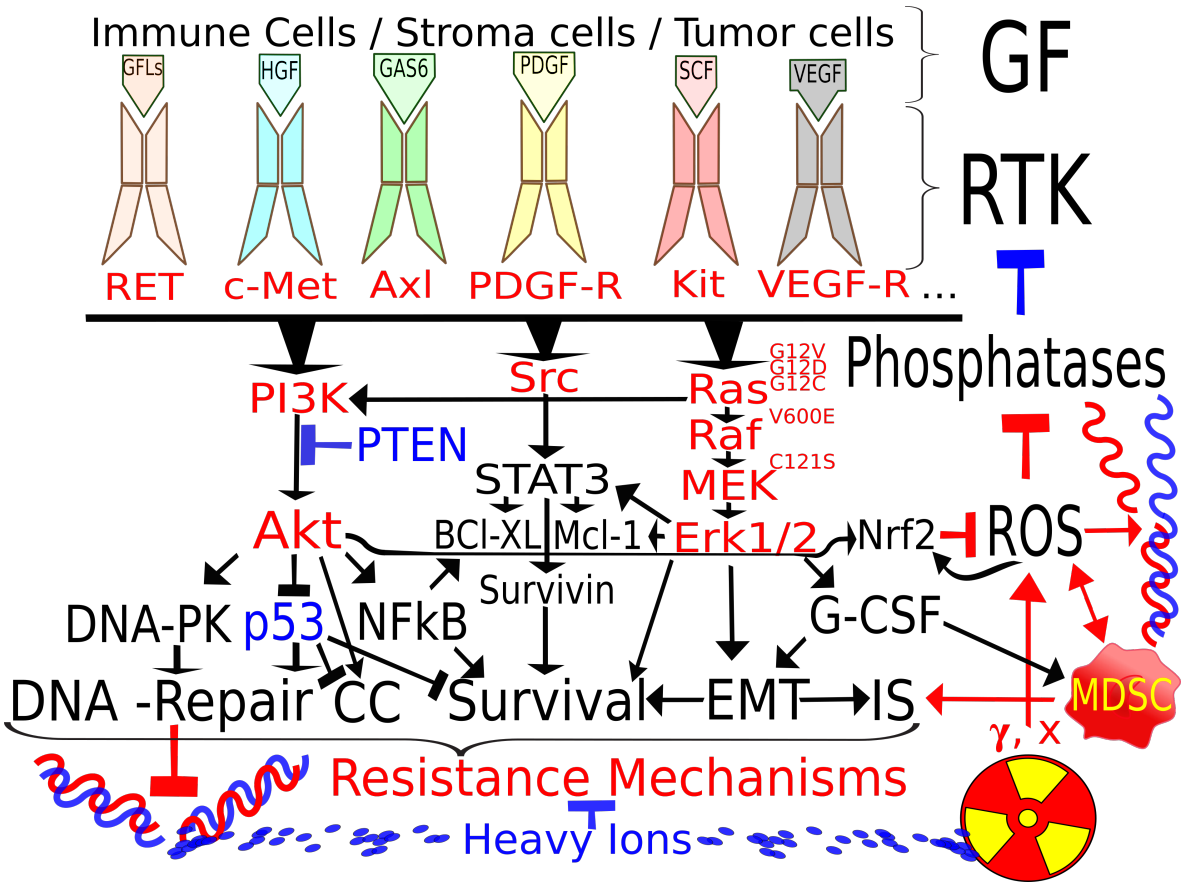

Figure 1: Brief overview of the interplay between radiation, molecular pathways and resistance mechanisms. Molecules in blue color are the two most crucial tumor suppressor genes, the phosphatase **PTEN** [54,55] and the key (but frequently mutated) transcription factor **p53** [3,56,57]. Depicted in black are key oncogenic molecules with their most frequent mutations [3] and ensuing treatment resistance mechanisms. **GF**: Growth factors [33,58]; Molecules in red are receptor (upper row) and non receptor kinases. **RTK**: Receptor Tyrosine Kinases [59], e.g.: Axl [60–62] **Met**, the receptor of the hepatocyte growth factor (HGFR) [63,64]; PDGFR [65], RET [66]; The following molecules are all (non receptor) kinases [67,68]: **PI3K**: phosphatidylinositol-3-kinases [69–74]; **Akt**: Protein Kinase B [72,75–78]; **Ras**: Rat Sarcoma [79,80], the first discovered oncogene [1,2], mutated in virtually all pancreatic cancers [81], some sarcomas [82] and others [3]. Worsening prognosis under SBRT conditions [83] and Y-90 radioembolization [84]. Targeting possible e.g. via MEK-Inhibition [82,85] and Reoviruses with radiotherapy [86]. **Raf**: Acronym for Rapidly Accelerated Fibrosarcoma, mutations very prevalent in melanoma [87–89]. **MEK**: Map Kinase which activates Erk (MAP-Kinase Kinase, MAPKK) [90,91]; **Erk1/2**: extracellular signal regulated kinases [92], also called **M**itogen **A**ctivated **P**rotein **K**inases - **MAPK** [93–97] The following three molecules are key transcription factors for treatment resistance: **STAT3**: Signal transducer and activator of transcription 3 [98–102,102–104]; **NFκB**: nuclear factor κ-light-chain-enhancer of activated B cells [105]; **Nrf-2**: Nuclear factor (erythroid-derived 2)-like [106]; The following three molecules are markedly anti-apoptotic: The Bcl-2 family [107] proteins BCl-XL & Mcl-1 [108] and the IAP [109,110] group protein Survivin [111–116]; The only cell on the scheme, known for immune suppression **(IS)**, promotion of metastasis [117] and induction of Epithelial Mesenchymal Transition [118] **(EMT)**: Myeloid Derived Suppressor Cells **(MDSC)** [30–32,102]. Which can be antagonized e.g. by inhibition of B-RAF [119] and PI3Kγ [120] with Gemcitabine [43,121] or by antagonizing **G-CSF**: Granulocyte-colony Stimulating Factor, a cytokine expanding these cells [122], leading to invasion and metastasis [123]. **ROS**: Reactive Oxygen Species are tumorigenic e.g. by oxidizing phosphatases [124] and driving inflammation [22], but are also critical effectors of x-ray irradiation [13,57,125]. Carbon Ion Irradiation additionally [126] leads to excessive amounts of double strand breaks [127] thus being 4-10 times more efficient than x-ray [13] and therefore relatively ignorant facing intratumoral heterogeneity [128].

4.1 in vitro

Encouragingly, oncogenic pathways in tumor cells, like PI3K[129], are important in inflammation[130] and immune suppression[24,120,131,132]. This reveals small molecule drugs[52,53] like kinase inhibitors[68] as ideally suited to integrate into combined treatments[52]. Considering figure 1, the branching point at the level of the RAS oncogene is upstream both of the Akt and Erk/1/2 pathways. So, it is tempting to see results upon pharmacological treatment of both pathways in parallel e.g. in cases of oncogenic RAS mutations[97]. This is one of the examples of combined targeted therapy[133,134], which is also under investigation in combination with x-ray irradiation[135]. The pathways in figure 1, and many more, are primarily considered in terms of direct effects of tumor cell inactivation. For immune mediated cell killing, other parameters are relevant, too. Some of these issues are now followed consecutively in the same order as presented in the results and discussion parts.

Phenotype changes

In order to find tumor cells, the immune system needs to recognize the tumor antigens arising from mutations or translocations. During cancer progression, the immune system interacts with the tumor both in terms of tumor promotion and desctruction, gradually enriching the tumor in cancer cells it can no longer find, a process termed immunoediting[136]. This is partly caused by a gradual decrease in MHC-I, the molecule responsible for immune recognition[121]. Therefore, loss of MHC-I is a treatment resistance mechanism in tumors[121,137]. Under radiotherapy conditions in mouse models, immunity was shown to be mediated by CD8+ T-cells, to recognize tumor cells *via* antigen presentation on MHC-I[138–140]. Therefore, the increase in surface presence of MHC-I is an attractive consequence of irradiation[13]. Phenotype changes of tumor cells after x-ray irradiation with respect to upregulation of MHC-I[141,142] and CD95[143,144] have been observed, now also including proton irradiation[145]. CD95, also known as Fas, is a death receptor that was shown to be involved with a select form of apoptosis, which can be immune stimulatory[146]. Carbon ions are here characterized for surface translocation of CD95 and MHC-I using CT26.WT murine colorectal carcinoma cells and the murine breast cancer cell line 4T1.

Autophagy induction

Autophagy has many functions in immunity and diseases including cancer[147], and is a point of intervention in cancer therapy for its dichotomous, partly cancer promoting roles[148,149]. Autophagy is a process leading to the secretion of ATP, which attracts immune cells to the tumor[150], which could also apply to radiotherapy[150]. Autophagy inhibition did show efficacy in combination with MEK inhibition to restore the exposure of Damage Associated Patterns, which could activate the immune system[151–154]. A most notable example which raised a lot of attention was the doubling of median survival in Glioblastoma patients to 22 months, when low dose Chloroquine was added to standard radiochemotherapy[155] - but the mechanism of activity of chloroquine is still to be clarified (see below). Autophagy was shown to be a consequence of both radiotherapy[156] and chemotherapy[157], resulting in surface translocation of the Mannose-6-Phosphate Receptor, which was shown to be involved in immune mediated tumoricidal effects[156,157]. Additionally, it has been shown that modulation of the autophagy process with chloroquine was able to increase the effects of radiation in a immune dependent manner, apparently by prevention of antigen degradation in the murine MCaK breast tumor model[158]. Consequently, it is tempting to see in which ways autophagy modulation by chloroquine could be useful, and how this could relate to heavy ion therapy.

Dendritic Cell Maturation

The activation status of dendritic cells is considered the deciding factor in radiation induced immune surveillance[139]. This has been shown to be dependent on cytosolic DNA sensing of DNA from dead tumor cells by STING - signaling in dendritic cells[46,159] also following irradiation[46]. TLR4 - signaling by radiation induced release of HMGB1[151] was also shown to have an influence[160], and other mechanisms of sensing apoptotic cells[161]. Direct irradiation of wild type dendritic cells has been shown to upregulate the activation marker CD70, but not CD80 on dendritic cells generated from monocytes from human donors, leading to enhanced T-Cell priming[162]. Others found an increase of CD80 on Dendritic Cells derived from murine bone marrow upon irradiation[163]. It was shown that costimulatory signals via CD80 to CD8+ T-Cells were important for the immunogenicity of cis-platin based chemotherapy[48].

Molecular Mechanisms of surface exposure

A rational pharmacological augmentation of radiotherapy will probably be a lot easier knowing the molecular mechanisms involved. Blocking MEK using the selective Inhibitor Trametinib could vastly increase expression of MHC-I on CT26.WT cells[164]. In cases where irradiation does not lead to a surface translocation of molecules relevant for dendritic cell activation or tumor antigen recognition by T-Cells, appropriate pharmacological management could potentially help to reinstate surface translocation of desirable molecules like MHC-I or CD95. On the other hand, care should be taken with pharmacological agents which eventually interfere in this process.

Epithelial Mesenchymal Transition

Epithelial Mesenchymal Transition (EMT) is a process which is critical for tumor progression and metastasis, making cells motile and invasive[165], and is also implicated in Cancer Stem Cell maintenance[29]. EMT is impairing immune mediated tumor cell lysis *via* induction of autophagy[166] and is a process contributing to the formation of the immune suppressive tumor microenvironment[167]. It is an inflammation driven process[167,168] and therefore a key candidate mechanism of treatment induced cancer progression[169], including classical photon therapy[170–174] and hyper-fractionated radiation[175], pointing to oligofractionation as a most likely more favorable method. Epithelial Mesenchymal Transition is one of the most crucial phenotype changes, and is therefore a key candidate for pharmacological management. As we will see, selective inhibition of one single pathway could potentially be sufficient to defuse this problem in many cases. Finding more relevant agents for management will be useful[176].

4.2 in vivo

As stated above, oncogenic pathways (figure 1) also reverberate in the tumor induced immune suppression and other hallmarks of treatment resistance (figure 4). For some key players of problems related to the tumor microenvironment, a short snapshot is given. This serves to explain the choice of drugs which are evaluated in the *in vivo* part of this thesis, which are described just after the features of TME related cells. Contrariwise to individual biomarkers, which can only apply to few if not single patients, immune mediated mechanisms described below are common schemes, deeply rooted in inflammation, and hence cancerogenesis.

Tumor Cell Death

Killing tumor cells is a central issue in cancer therapy and can be mediated by different cell death modalities, e.g. apoptosis or necroptosis[149] and others[177]. Irradiation also can invoke tumor cell death e.g. by necroptosis, extrinsic (CD95, DR5) and intrinsic (p53) apoptosis and cell cycle arrest[57]. This can be hampered in cancer e.g. by genetic aberrations (deletion / mutation of p53 in 50 % of cancer cases in general, 90 % in ovarian cancer in particular[3]), which constitutes one of the hallmarks of cancer[29]. Radiation can also induce anti apoptotic molecules (see figure 1 and 2)[101] even at very low radiation doses in the range of CT scans (Survivin)[115]. Reinstating apoptosis is a desirable effect e.g. of Kinase Inhibitors[68]. Good success was seen clinically with stereotactic beam radiation therapy (SBRT) and the apoptosis inducing Tyrosine Kinase Inhibitor Sunitinib[178,179], which is directed e.g. against VEGF-R, Kit and PDGFR[180]. Levels of the immune suppressive MDSC were used as a readout pre-

dictive for therapy success[181]. A key point in tumor cell death modalities[177] is that tumor cells dying apoptotically appear to be more efficient in inducing antigen cross presentation by dendritic cells. Induction of apoptosis is a key point in anti tumor immunity, contrariwise to cell death induced by necro(pt)osis[161,182]. Here, we first characterize apoptosis induction via combined and specific targeting of MEK and c-Met in CT26.WT cells. Then we expand this approach to CT26.WT, 4T1 and B16-F10 tumor cells with irradiation and pleiotropic agents, as well as treating physiologically normal HMVEC cells.

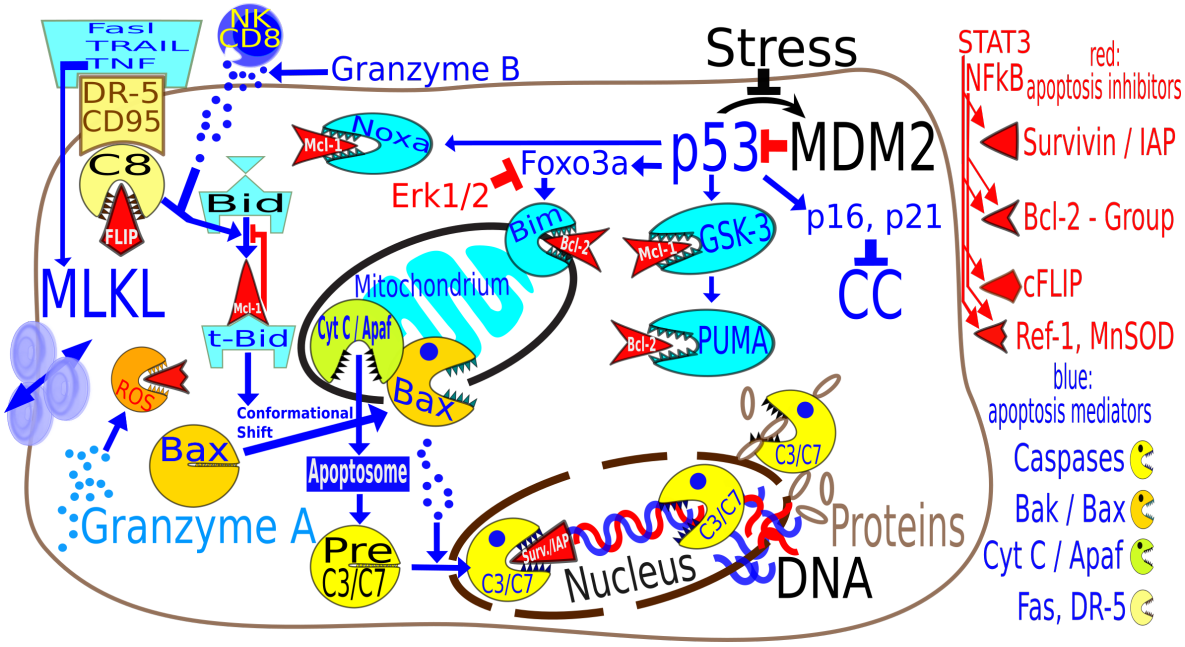

Figure 2: Depiction of cell death mechanisms[183], which show how molecules depicted in figure 1 can interfere with tumor cell death. **Extrinsic apoptosis** can be triggered by death receptors[184,185] like **DR-5**[185,186] and **CD95**[143] *via* **caspase 8**[152]. **Intrinsic apoptosis** follows stress responses, which can lead to a buildup of **p53**[187,188], with the DNA-damage response being most relevant for radiotherapy[189,190]. Activation of p53 leads e.g. to GSK-3[191] dependent expression of PUMA[192]. So, the apoptosis 'inducer' p53 is to a significant extent doing what pro apoptotic pharmaceuticals do: Antagonizing anti - apoptotic **BH3 only proteins (e.g. Bcl-2, Bcl-XL, Mcl-1)**[107], **Erk1/2**[193] and other molecules shown in figure 1. Another way to apoptosis induction is stress of the endoplasmatic reticulum (ER-Stress)[194], which also can lead to a buildup of the pro apoptotic molecule Bim[195]. Adding to apoptosis, p53 is steering a magnitude of other cell processes[188]. It also can stall the cell cycle (CC) and clearly has functions beyond these activities[196]. Both intrinsic and extrinsic apoptosis are converging on caspases 3 and 7. Granzyme A is activating a different cell death program dependent on Reactive Oxygen Species (ROS), which is deployed e.g. by Natural Killer Cells, and also leads, as apoptosis does, to phosphatidylserine outer membrane translocation **(Annexin-V - positivity)**[197]. **Necroptosis** is a non apoptotic cell death modality[198] triggered e.g. by TNF[199], leading to a homotrimer[200] of Mixed Lineage Kinase Like (MLKL, blue donut trimer to the left)[201,202], perforating the cell membrane **(DAPI positivity)**[200,203]. Necroptosis has key roles in inflammatory diseases including rheumatoid arthritis[204], with ROS being involved[205]. **Ferroptosis**, not depicted here, is also deemed pro inflammatory and driven by iron, which leads to hydroxyl radicals via Fenton reactions, resulting in lipid peroxidation[206]. **Autophagy** can be seen as a mechanism integrated between the different cell death programs[207] and therefore shapes how these programs lead to immunological responses and cancer metastasis[208]. Conclusively, the cell death pathways play a crucial role in immunity in general[152], steer how radiotherapy is influencing the immune system, and are therefore relevant for therapy success[209].

The Tumor Microenvironment

The cell intrinsic issues mentioned above integrate *in vivo* in the tumor microenvironment (TME) in a bidirectional way. The tumor microenvironment fuels the above mentioned issues like EMT, invasive growth and apoptosis resistance. On the other hand, tumor cells manage to attract cells from the bone marrow to support their growth[210]. The individual conditions in the TME are now proven by big data efforts to decisively define disease prognosis[211,212], metastatic dissemination[27,117], resistance to chemotherapy, targeted treatments[213], and radiotherapy[214]. Consequently, tumors are increasingly seen as complex organs[29], giving rise to cancer as a systemic disease. Encouragingly, the properties of the cellular constituents become increasingly known[215], and offer useful mounting points for therapeutic interventions. The *in vivo* efforts in this thesis aim to rewind some of the tumorigenic processes which are prevalent in the TME per se, wich can be made worse and improved by radiation. Cancelling out the adverse effects of radiation pharmacologically while optimizing the favorable effects with radiation (fractionation, dose, quality [particles, photons]) could be a promising strategy, here exemplified in the extremely aggressive, highly metastatic 4T1 model. Some key players of the TME are briefly sketched out in the following, to explain some pharmacology deployed in the field and attempts to approach these issue(s) this thesis.

Myeloid Cells

Myeloid cells are mostly discussed as Myeloid Derived Suppressor Cells (MDSC) and Tumor Associated Macrophages (TAM)[216–220], are key players in cancer metastasis by immune independent mechanisms[216,221], neoangiogenesis, as well as systemic[27] and local immune suppression[28,117]. They are vastly expanded by cytokines, like (also radiation induced)[170] G-CSF[221–223] even to the point of leukemia - like conditions[223]. The expanded MDSC are attracted to the tumor e.g. by VEGF, as a consequence of tumor hypoxia related HIF-signaling[224]. The function they are exerting is driven by growth factors like VEGF (both a cytokine and a growth factor), PDGF, reactive species of nitrogen and oxygen[32].
Gemcitabine is a chemotherapeutic used concurrently with carbon ions e.g. in pancreatic cancer[44] and was shown to be active against MDSC. This might explain parts of the efficacy of this combination[43]. There have been incredibly good results in the notoriously aggressive 4T1 model, selectively antagonizing Myeloid Suppressor Cells (MDSC), leading to long term surviving mice. However, success came with intermediary but great animal weight loss, revealing both an important strategy and a need to be aware of toxicity in order to enable this promising approach for patients[225].

Besides the notorious MDSC, which are recruited to the tumor in large numbers *via* radi-

ation[226] Tumor Associated Macrophages (TAM)[217,218,220,227] are also immune suppressive myeloid cells[217], frequently present in Tumor Microenvironment in a diversity of tumor entities[228], and contribute to neovascularization after radiotherapy[219]. They are also involved in raising the fibrotic problems indicated below, e.g. supplying the tumor microenvironment with PDGF[219]. However, they can be divided into tumor promoting M2 and tumor suppressing M1 macrophages - termed macrophage polarization, which is a key factor in probably all cancer therapies[227] . Crucially, this polarization appears to be flexible, allowing to turn M2 into M1 macrophages, which could support therapy efforts. Low radiation doses, which might not be very relevant for the usually higher doses in SBRT, can lead to a favorable M1 polarization[229]. Contrariwise, therapeutic radiation could foster M2 polarization[219] urging to adress this issue. In the 4T1 - Model, the tumor microenvironment is densely populated by myeloid cells (MDSC/TAM) and very scarcely by T-Cells, reflecting the treatment resistance mechanisms indicated above[224,230]. Therefore, appropriate pharmacological macrophage management either by depletion or repolarization is seen with considerable hope and has already shown efficacy[227]. Depletion of both MDSC and TAM with the shared marker Gr-1 was able to decrease local recurrence after radiotherapy[231]. However, chosing the right targets appears to be a crucial issue, as some treatment measures also can even enhance metastatic spread and tumor growth[232].

Fibroblasts

are long known to radiation oncologists for their role in radiation fibrosis, a dreadful late complication after radiotherapy[233,234]. More recently, it was revealed that cancer associated fibroblasts in the tumor stroma strongly contribute to treatment resistance as a barrier to chemotherapeutics and immune cells[235], being severely immune suppressive even when their prevalence in the tumor stroma is as low as 2 %[236]. Even though most therapy measures to date have been thought to be directed just against tumor cells, and tumor cells per se can also be strongly immune suppressive[237], in the difficult KPC mouse model of pancreatic cancer, *selective depletion* of FAP+ fibroblasts *only*, using CAR-T-Cells directed against FAP (and hence no tumor cells), abrogated tumor growth[235]. Critically problematic for radiotherapy, fibroblasts entirely retain their immune suppressive capability even after high SBRT - type radiation doses[238], and radiation apparently can exacerbate metatasis enhancing properties of fibroblasts[239].

So, we have clear evidence that it could be very helpful to antagonize fibroblasts. It currently is unclear if we can have clinically approved measures directed against fibroblasts per se, and the cellular interventions which have proven successful in mouse models[235] require highly trained experts to be put into action, currently limiting a desirable broader use.

Potentially, it might be possible to target the inflammatory processes leading to fibroblast

formation, which somehow mimic the processes leading to immune suppression[240]. Another option is to intervene in fibroblast - mediated signaling, which can raise resistance against targeted drugs[241]. For example, it was shown that targeting of PDGF-R allowed to strongly ameliorate the induction of radiation induced fibrosis[242], in line with a report on PDGF-Rβ as a marker of stromal activation[243].

T-Cells, specially CD8+ T-Cells, are key players in radiotherapy efficacy, and needed in the tumor to augment the efficacy of radiotherapy[47,160]. The depletion of MDSC after high dose irradiation in the CT26 model was shown to be dependent on CD8+ T Cells[160]. Some tumors are devoid of T-Cells[244]. To change this is apparently a decisive effect of radiotherapy[245,246]. Other cells influencing the tumor microenvironment[214,247,248], like immune suppressive Mesenchymal Stromal (MSC) cells[249,250], which can be vastly increased in numbers in a CCL2[251] dependent manner after irradiation e.g. to fuel neoangiogenesis[252], and can expand MDSC via HGF/c-Met[253], cutting down on radiotherapy efficay.

Neutrophils[254], the most prevalent leukocytes, are still not clearly discernible from MDSC and thus add to the MDSC conundrae[255].

Pharmacological Management

Finding an integrated approach to match the challenges discussed above may seem like an arduous task, and is taken up by international structured efforts[256]. However, many molecules initially thought as target structures in cancer cells are also involved in tumor relevant systemic processes (see above) and at the same time key features of FDA approved[257] targeted drug regimens (see figure 3). Using the hitherto underestimated versatility of small molecule drugs in immune modulation[52], a combination of three pharmacological entities was applied to a small panel of representative tumor cell models and the murine 4T1 model of rapidly metastasizing breast cancer, which invokes a massive expansion of MDSC[258] and is highly resistant to therapy approaches[225,259]. Approaching this way has certain benefits of small molecules:

Clinical experience with the drugs of choice is key for clinical translation.

Lower Toxicity, as immune modulation is different from direct tumor targeting.

Target diversity leaves tumors fewer options exploiting backup pathways.

Off target on purpose effects enable a multimodal approach (see figure 3).

The description below explains how the treatment modality of choice is thought to integrate the challenges described above.

Cabozantinib (XL-184) is a Tyrosine Kinase Inhibitor (TKI)[260], approved for medullary thyroid cancer, and shows activity in a diversity of Tyrosine Kinases (including all of the Receptor Tyrosine Kinases shown in figure 1), amongst them RET (5.2 nM), MET (1.3 nM), VEGFR2 (0.035 nM), KIT (4.6 nM)[261,262], Tie2 and PDGF-R[257]. Despite it is viewed mainly as a anti vascular agent for its VEGFR2 - inhibiting properties[248], targeting of VEGF also strongly reduced the prevalence of myeloid cells in the 4T1 model[224]. Adding to a reduction of myeloid cell numbers, XL-184 was shown to enrich the tumor microenvironment with both CD4 and CD8 T-Cells, and did demonstrate immune mediated activity in the MC38 model of colorectal carcinoma[263]. Its reported activity against PDGF-R could potentially be of interest to antagonize (also radiation induced) fibrosis[242]. XL-184 was shown to derail the cancer promoting crosstalk between fibroblasts and tumor cells in a HGF dependent manner[264], and therefore potentially could reduce the MET dependent expansion of MDSC via MSC derived HGF[265]. This could be highly attractive in radiotherapy, as MSC can be recruited to tumors via irradiation[252], where they could contribute to the buildup of fibroblasts, which are, as stated above, key players in treatment resistance[266,267]. Adding to HGF, Angiopoetin is another growth factor secreted by MSC which can expand myeloid cells *via* the

Tie2 receptor[249], another kinase target of XL-184[257]. Sunitinib was shown earlier to have useful immune modulatory properties, and is in so far successful studies to augment SBRT. However, Sunitinib is not an inhibitor of c-Met. This property can be particularly useful for radiotherapy, as HGF[268] and PDGF[269] signaling is kind of a hypoxia induced backup pathway for angiogenesis[268].

Most crucially, c-Met is shown by a huge body of evidence to be relevant across practically all cancerous disease entities, and highly involved in disease progression[270]. Targeting c-Met specifically was shown to induce DNA damage related senescence in a radiosensitizing manner[271], rendering this drug target particularly interesting for heavy ion radiotherapy.

The c-Kit - Inhibition by Imatinib, which shares a lot of kinase targets with XL-184 (c-Kit, PDGFR, VEGFR...)[257], was shown to antagonize regulatory T-Cells via suppression of indoleamine 2,3 - dioxygenase (IDO)[272], a molecule involved in MDSC recruitment in a Treg dependent manner[273]. Conclusively, also in highly tumor targeted agents, there is a lot of room for immune modulatory activities (see Figure 3)[52].

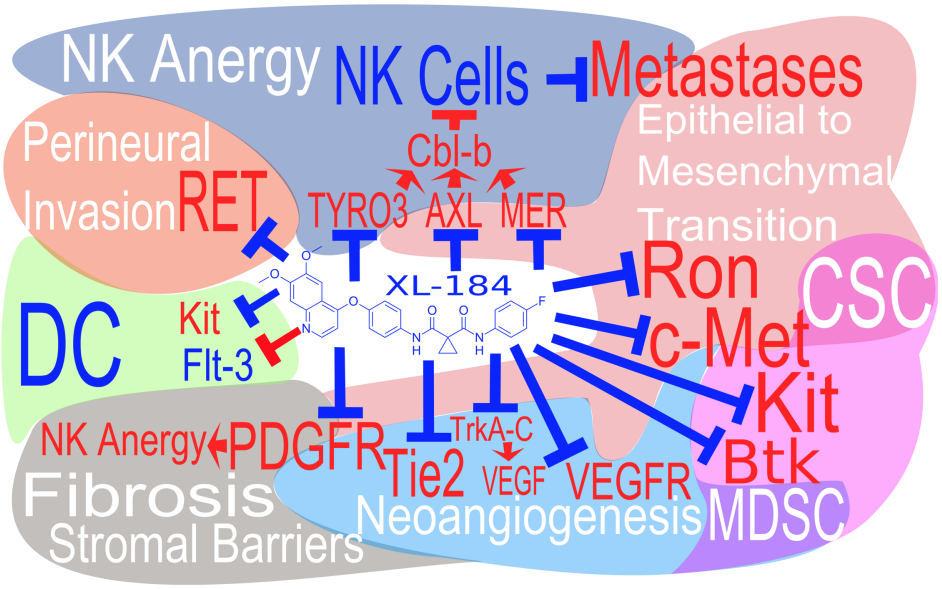

Figure 3: XL-184 (Cabozantinib) did show activity against about 80 kinase targets in a cell free assay[257] and was shown to be immune activating[263,274]. Here depicted is a selection of its targets mapped to possible mechanisms. (Tyro3, Axl and Mer)[275], Flt-3[276], Ron[277], PDGFR[242], Tie2[278], RET[66], c-Met (http://www.vai.org/met/)[279,280], Btk[281,282]. However, the activity against Flt3 could hamper DC function[283].

CDDO-Me is a semisynthetic triterpenoid[284] with a multitude of tumoricidal properties[284,285], acting pro apoptotic in many tumor cells and preventing epithelial mesenchymal transition[286]. It is a strong activator of Nrf-2, and was shown to block immune suppressive functions of MDSC in some mouse models and a small patient group in a non toxic manner[287]. It was demonstrated to repolarize tumorigenic M2 to tumoricidal M1 macrophages[288]. As radia-

tion induced long-term intestinal adverse effects have surpassed inflammatory bowel disease in incidence[289], the high efficacy against radiation induced inflammation and tissue damage is particularly interesting[290–292]. With inflammation being a key driver of carcinogenesis[24], the efficacy in reducing radiation related carcinogenesis[293] and x-ray related fibrosis[294] is revealing CDDO-Me et al. very interesting for improving radiotherapy. CDDO-Me did show anti metastatic properties in the 4T1 model as liposomal formulation[295]. However, as a note of caution, Nrf-2 inducers could potentially also protect tumor cells from radiation, potentially necessitating patient stratification[296].

Chloroquine is now being used as an anti inflammatory drug against arthritis with very acceptable side effects[297]. It was able to double the median survival of glioma patients together with chemoradiation[155] and improved the immunogenicity of radiation in the murine MaCaK model of breast cancer[158]. It also was shown to improve CD8+ mediated immunity[298,299]. Chloroquine was shown to ameliorate the metastasis-enhancing properties of fibroblasts in the 4T1 model by anti inflammatory mechanisms[239], and was shown to confer Notch-dependent tumor vessel normalization[300]. However, despite reports of anti metastatic activity in the 4T1 model[239,301], Chloroquine did not show impressive activity in 4T1 tumor growth with or without radiation[302]. Nevertheless, as a safe adjunct component its benefit could outweigh the risks.

5 Results

5.1 Phenotype changes of tumor and immune cells

The murine tumor cells chosen for this experiments are hallmark model systems in immunology, and extensively characterized. The U2OS cell line stably transfected with LC3-GFP-RFP enables to follow location and function of autophagic vesicles by microscopy. The murine tumor cells used in this thesis represent a spectrum of different properties with respect to the p53 mutation status, the mutation load and hence their immunogenicity. CT26.WT cells overexpress wild type p53[303], B16-F10 cells express p53 mutated in the DNA binding region and mutated PTEN[304], whereas 4T1 cells are p53 protein negative[305]. About half of tumors express mutated p53. Given the role of p53 in apoptosis induction (see figures 1 and 2) and immunity[306], p53 status is a central determinant in treatment responses[306]. This section is mainly thought to evaluate the phenotype changes of these test systems to different treatment cues.

5.1.1 Exposure of MHC-I and CD95 after x-ray and carbon irradiation on CT26.WT cells

In the murine CT26.WT colorectal carcinoma cell line, which is triploid in double mutated K-Ras (V8M, G12D)[303], x-ray - irradiation (figure1) did result in an increase in surface presence of MHC-I (figure 4). Irradiation with carbon ions did lead to a smaller increase in surface translocation, which demonstrated a saturation at rather small doses of approximately 2-3 Gy (figure 5). The smaller tendency of carbon ion irradiation to induce surface translocation compared to x-ray was also found for the death receptor CD95, and was also seen in many other FACS - Samples, of which representative examples are shown.

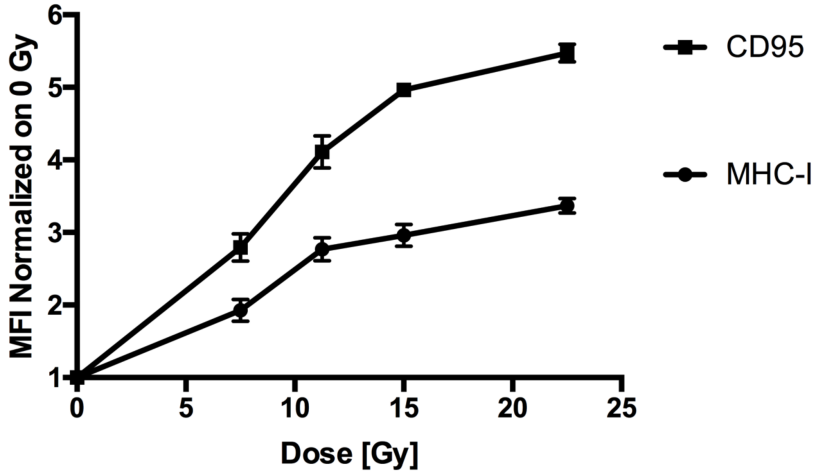

Figure 4: Exposure of MHC-I and CD95 on CT26.WT cells 24h after x-ray irradiation (n=3). Error bars represent the standard deviation of three measurements from one representative experiment.

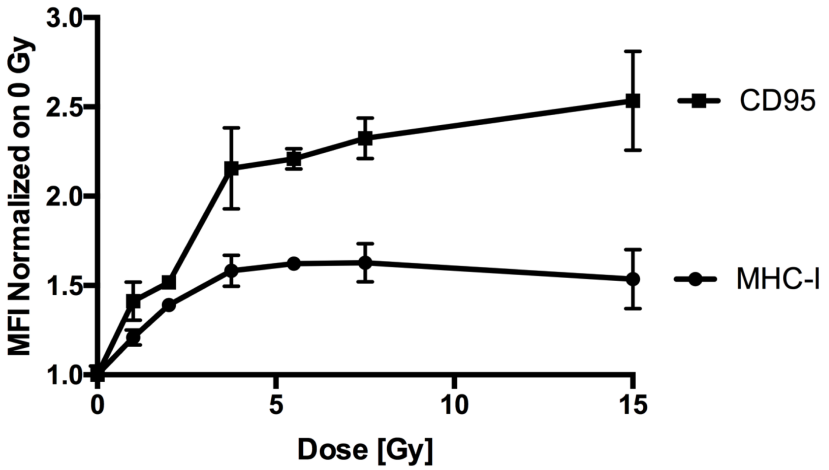

Figure 5: Exposure of MHC-I and CD95 on CT26.WT cells 24h after carbon ion irradiation (n=3) The LET was 325 keV/μm. Error bars represent the standard deviation of three measurements from one representative experiment.

5.1.2 Exposure of MHC-I and CD95 after x-ray and carbon irradiation on 4T1 cells

In the 4T1 cell line, x-ray irradiation did not lead to a surface translocation visibly different from carbon ions. However, a smaller number of samples was screened for the 4T1 cell line. In comparison to the CT26.WT cell line, also in other x-ray experiments, the surface translocation appeared to be generally rather small on the 4T1 cell line (figure 6).

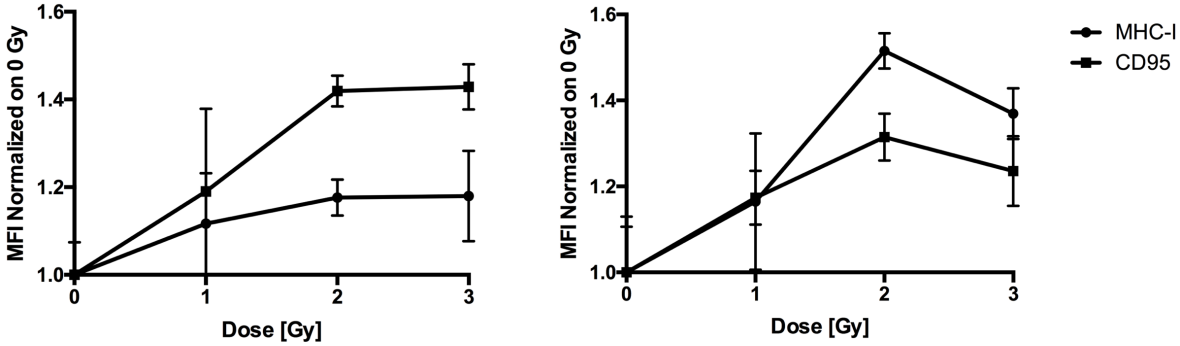

Figure 6: Exposure of MHC-I and CD95 on 4T1 cells 24h after x-ray- (left) and carbon ion irradiation (right, LET= 325 keV/μm). Error bars represent the standard deviation of three measurements from one representative experiment.

5.1.3 Induction of autophagy in U2OS cells via x-ray and carbon ions

Autophagy is a process inducible via irradiation[156] as well as chemotherapy[157], and contributes to the immune mediated effects of these standard therapy measures. Therefore, it is attractive to have access to methods to explore autophagy. The effect of 10 Gy X-Ray irradiation after 24 h is here demonstrated with U2OS - cells of human osteosarcoma, transfected with LC3 fused to GFP and RFP. To the best of my knowledge, this test system has not been subjected to irradiation so far. Acidification of the induced autolysosomes upon autophagy induction leads to a quenching of the green fluorescence, with the red signal remaining intact, visible per microscopy (see red arrows on figure 7). With carbon ion irradiation, no increase in red spots could be seen with doses ranging from 1 to 30 Gy after one experiment.

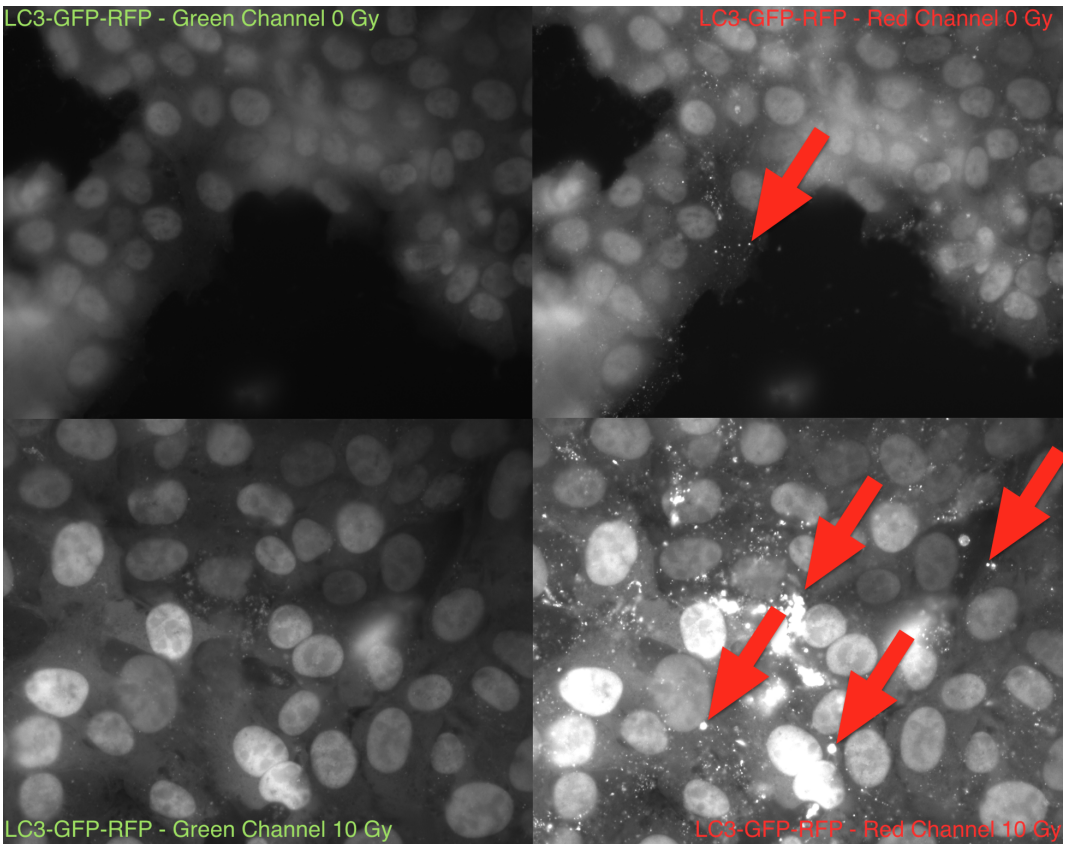

Figure 7: Induction of Autophagy in U2OS cells transfected with LC3-GFP-RFP 24 h after 10 Gy of x-ray. The irradiated sample (lower right) does show an increased abundance of acidic vesicles visible only in the red channel (red arrows). In control samples, fewer acidic vesicles are visible (upper right). The signal in the green channel is quenched as described in the text in the case of autophagically active vesicles. The cells were kindly provided by Oliver Kepp from the Group of Guido Kroemer, UMR1138 équipe 11, Centre de Recherche des Cordeliers, 15 Rue de l'École de Médecine, 75006 Paris France.

5.1.4 Switch from mesenchymal to epithelial phenotype via MEK - Inhibition

MEK is a crucial MAP Kinase and therefore a central signaling molecule in tumor cells (see figure 1), and therefore a candidate drug target for combination therapies. Treating CT26.WT cells with the MEK inhibitor Trametinib, a change to an epithelial phenotype was noted (figure 8), and found to be in line with publications in other cells, referring to cells with mutated BRAF subjected to MEK inhibition[307] and KRAS-mutated cells to Erk1/2 inhibition[308].

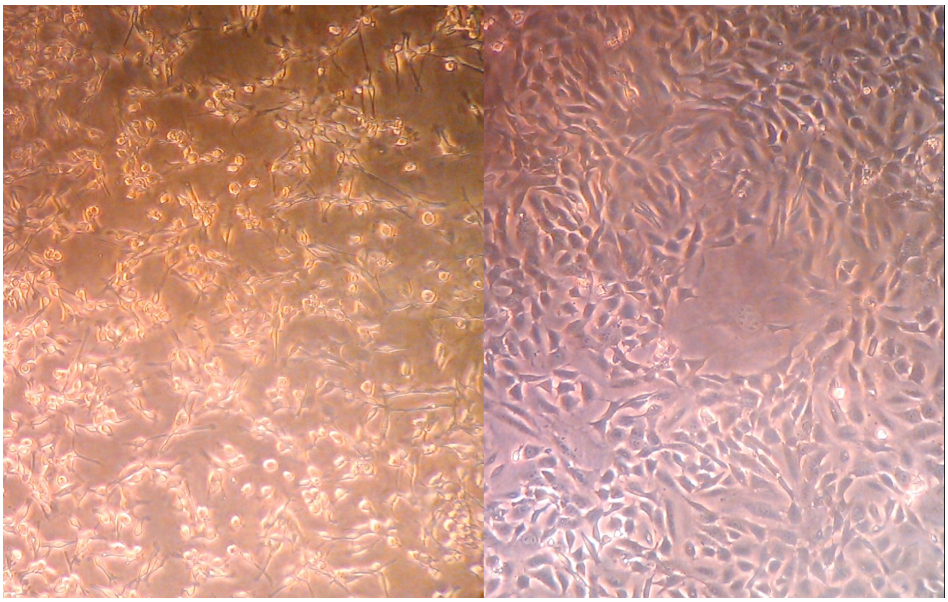

Figure 8: Switch from mesenchymal (left) to epithelial (right) phenotype via MEK - Inhibition in CT26.WT cells after 24 h with 100 nM of Trametinib.

5.1.5 Apoptosis induction via X-Ray and combined Inhibition of MEK and Met

CT26.WT cells were incubated for 20 h with each 100 nM of Trametinib, a selective Inhibitor of MEK (see figure 1), and EMD1214063, a selective inhibitor of c-Met [309] (see figure 1). Cells were irradiated with 10 Gy and after 24 h analyzed with DAPI and Annexin-V - staining for evaluation of necrotic and apoptotic cells. Despite the response at this time point was quite low, with only few detectable apoptotic cells, it is suggesting an additive effect of the treatment measures in this preliminary experiment (figure 9).

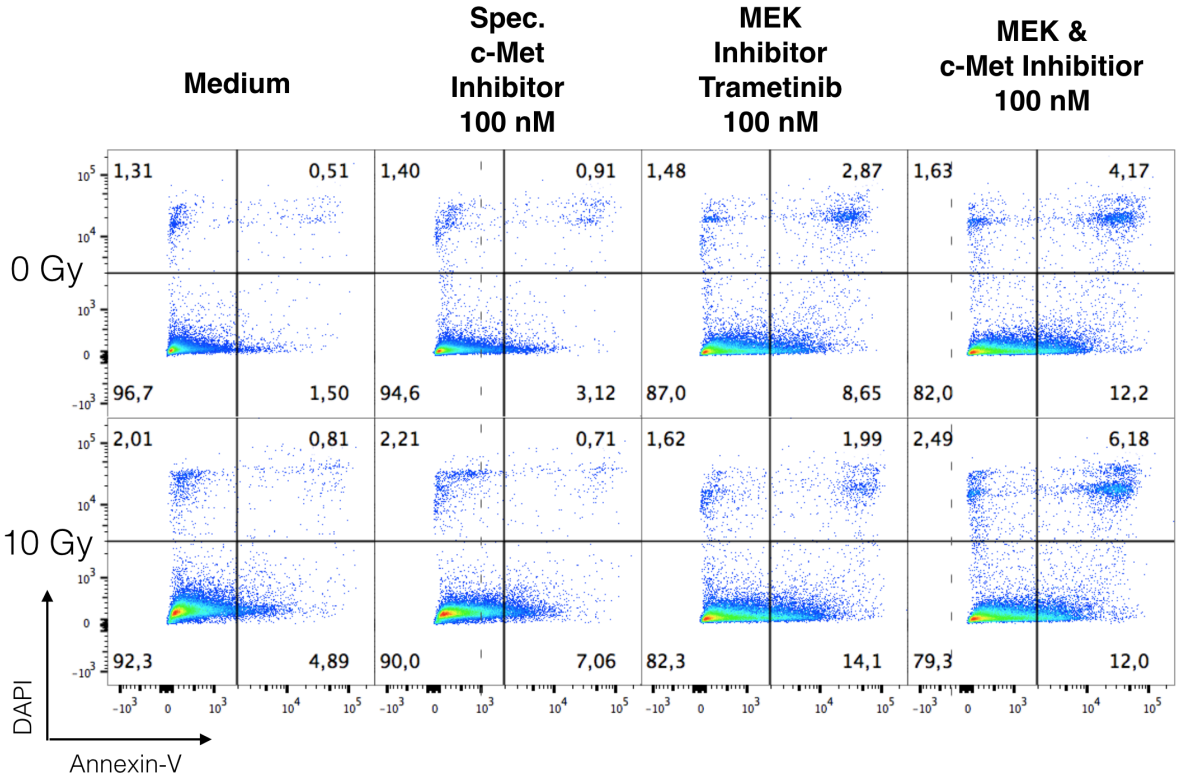

Figure 9: FACS plots of DAPI and Annexin-V stained CT26.WT, incubated as indicated, irradiated with x-ray after 24 h and worked up for analysis after media change and 24 h of further incubation.

5.2 Translational approach, in vitro

The above mentioned experiments refer to single molecules and readily discernible mechanisms, using selective inhibitors. In contrast, the drug regimen chosen for the in vitro- and in vivo experiments is highly pleiotropic - a concept termed polypharmacology[4]. The results are characterized in the following via DAPI / Annexin - V - staining in three tumor cell lines and human microvascular endothelial cells representing normal tissue, western blot analysis of cancer cell survival pathways and surface exposure of immune relevant molecules on CT26.WT cells.

5.2.1 Induction of Cell Death in CT26.WT tumor cells, x-ray and carbon ion experiments

CT26.WT cells were incubated with Chloroquine, CDDO-Me and XL-184 at the indicated concentrations for 20 h, irradiated with the indicated doses and submitted to FACS - Analysis after 24 h. Adding to an apparently high background of necrotic Propidium Iodide+ cells, upon combination of all three drugs a population of cells positive both in Propidium Iodide and Annexin-V staining emerged with and without irradiation (figure 10).

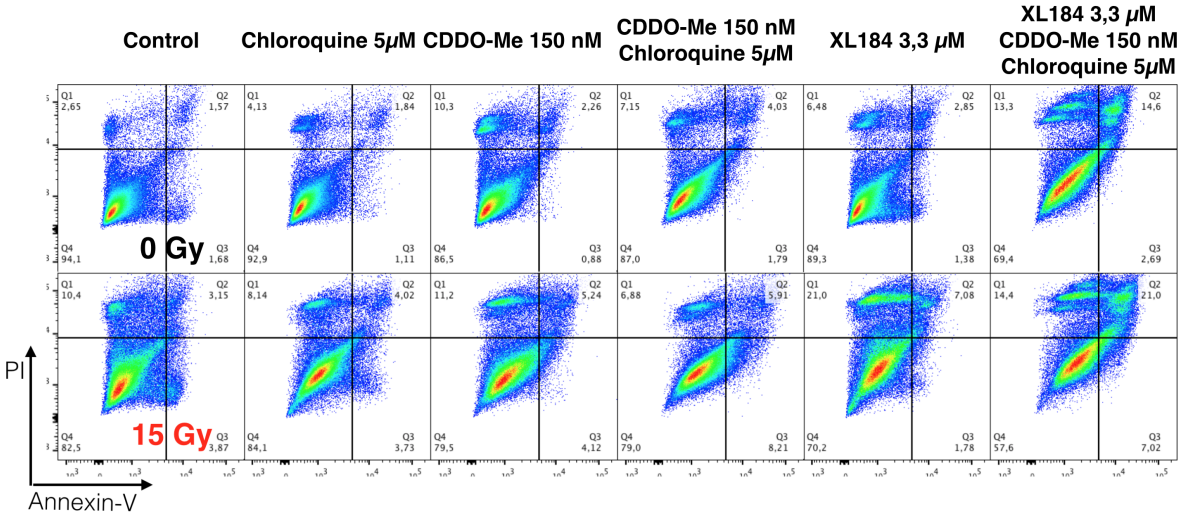

Figure 10: FACS - plots of Propidium Iodide (PI) / Annexin-V stained CT26.WT cells after drugging with the indicated drugs and x-ray irradiation, concentrations and radiation doses.

In a representative experiment with carbon ion irradiation, a shift from necrosis to apoptosis was also visible under influence of the drugging regimen with the same drug concentrations as in the x-ray experiments (figure 11).

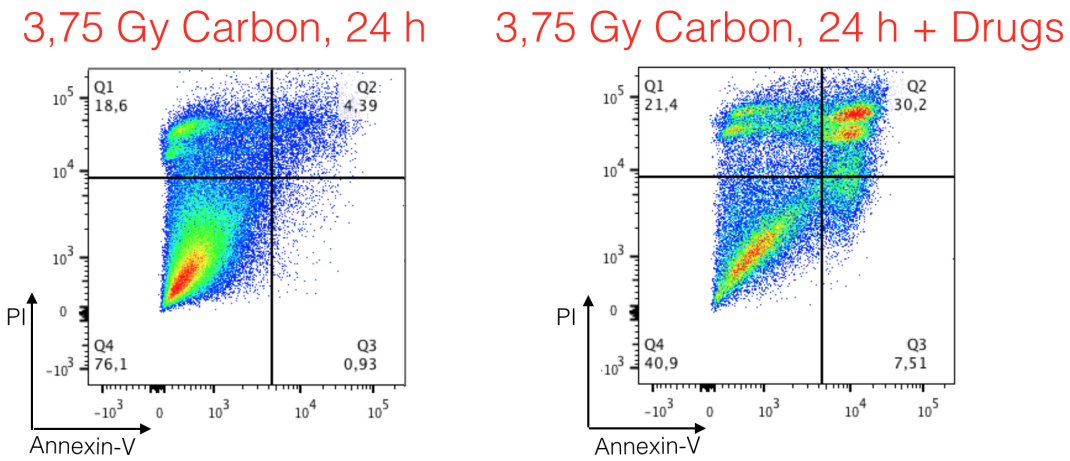

Figure 11: FACS - plots of Propidium Iodide / Annexin-V stained CT26.WT cells after drugging with Chloroquine, CDDO-Me and XL-184 for 20 h as above and irradiation with carbon ions. The LET was 168 keV/μm.

5.2.2 Induction of Cell Death in B16-F10 tumor cells (x-ray)

B16-F10 cells are a frequently used model for melanoma in preclinical research, and able to undergo apoptosis. B16-F10 cells were treated as the CT26.WT cells, with the difference of a lower concentration of XL-184 of 2.5 μM. Despite this dose reduction, a pronounced response was found also in this cell line, indicating late apoptosis. The combination of all three drugs also here led to the presence of apoptotic cells in a way much more pronounced in comparison to the individual treatments (figure 12).

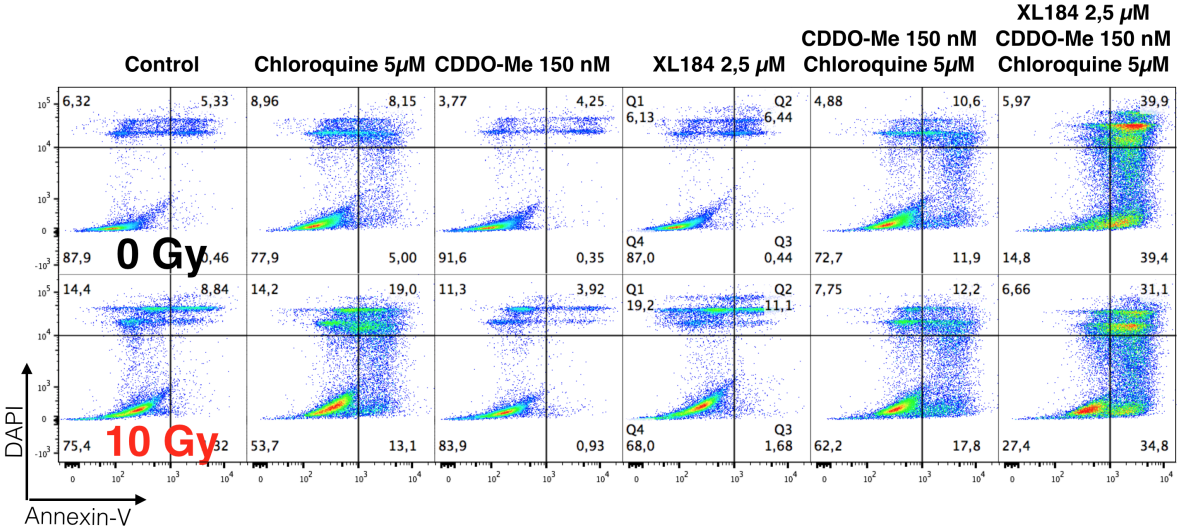

Figure 12: FACS - plots of DAPI / Annexin-V stained B16-F10 cells after drugging and irradiation with the indicated drugs, concentrations and radiation doses. Measurement took place 24 h after irradiation as for the other cell lines. The data was raised with technical assistance from the master students Dominique Tandl and Tobias Schulze (TU Darmstadt).

5.2.3 Induction of Cell Death in 4T1 tumor cells (x-ray)

4T1 cells are a frequently used model for metastatic breast cancer in preclinical research, and are not prone to undergo apoptosis. Despite the 4T1 cells were treated like the B16-F10 cells, the response was different to the results above. Annexin-V positive cells were barely visible in any of the treatment condition neither with nor without radiation. The most pronounced response resulting in DAPI positive necrotic cells was seen with XL184 and irradiation without other drugs. In the experiment, this treatment group was part of a duplicate with a similar result (figure 13).

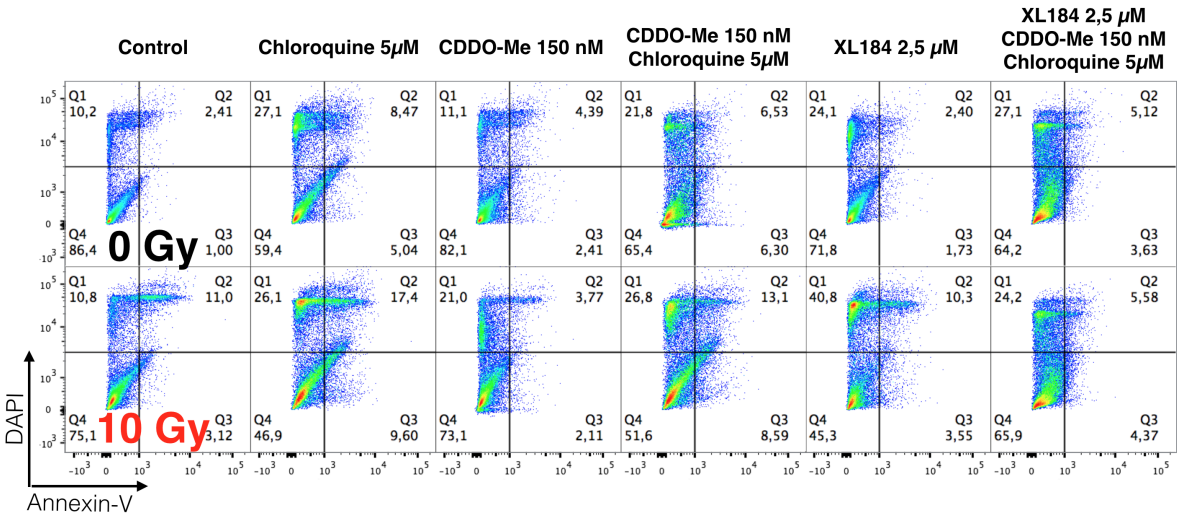

Figure 13: FACS - plots of DAPI / Annexin-V stained 4T1 cells after drugging and x-ray irradiation with the indicated drugs, concentrations and radiation doses. Measurement occured 20 h after irradiation.

5.2.4 No Induction of Apoptosis in HMVEC normal tissue cells

Human microvascular endothelial cells (HMVEC) of low passage number were treated similar to the B16-F10 and 4T1 cells using 2.5 μM XL-184. In all treatment groups including the samples irradiated with 10 Gy x-ray, cell death as assessed via DAPI / Annexin-V staining remained above below 5 %, revealing no apparent signs of toxicity (figure 14).

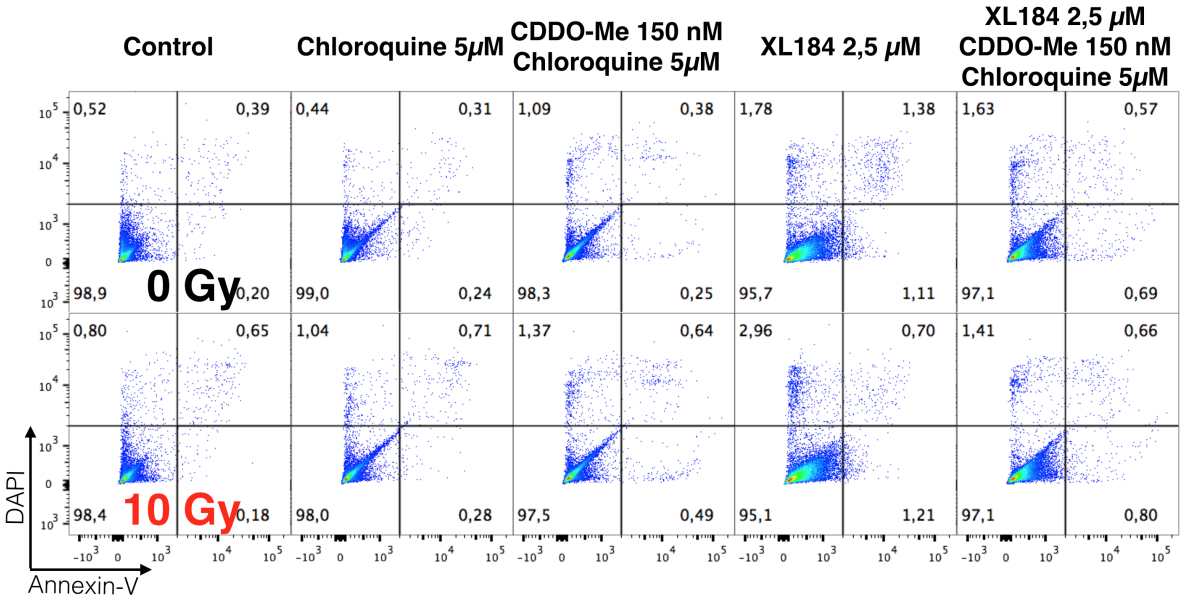

Figure 14: FACS - plots of DAPI / Annexin-V stained HMVEC normal tissue cells after irradiation with x-ray and treatment with the indicated drugs, concentrations and radiation doses.

5.2.5 Anti apoptotic and oncogenic molecules in CT26.WT cells

CT26.WT cells were incubated with Chloroquine (CQ) [5 μM], CDDO-Me [150 nM] and XL-184 [3.3 μM] for 44 h and subjected to Western Blotting. Values were normalized to untreated cells, blotting was carried out in technical triplicates with material from one experiment. Apparently, XL-184 has an effect of diminishing Erk/1/2 phosphorylation levels, whereas CDDO-Me was reducing the abundance of molecules known for derailing apoptosis, namely Bcl-XL, Mcl-1 and Survivin. There was also a visible but less pronounced effect of CDDO-Me on the abundance of phosphorylated Akt and phosphorylated STAT3. The lowest values of 20 % in the densitometric measurements appeared to be close to background levels. Densitometric values were normalized to the signal of vinculin as house keeping gene, and the values of the drugged cells were normalized to the values of untreated cells (figure 15).

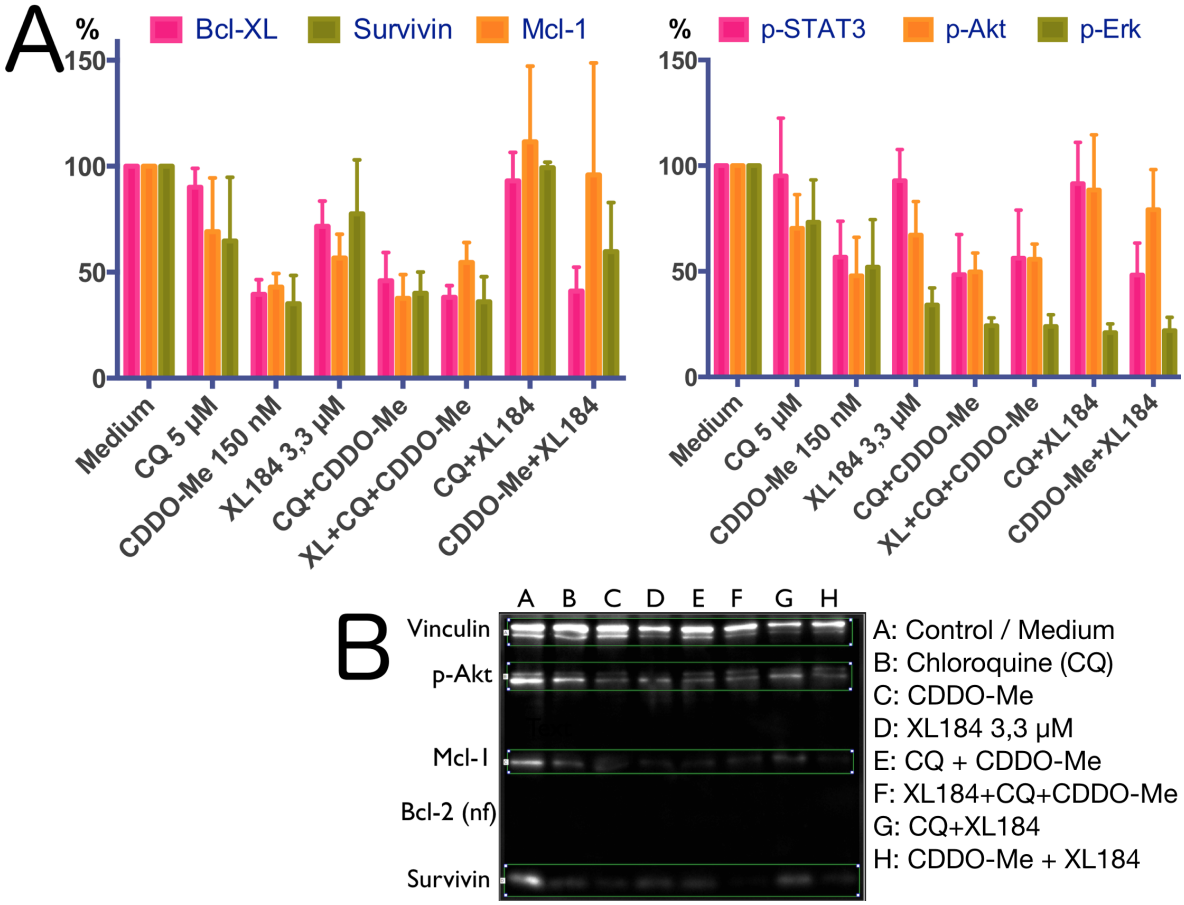

Figure 15: A: Densitometric data collected from western blots performed in technical triplicates from one experiment. CT26.WT cells were drugged for 44 h as indicated. B: Exemplary western blot with 7.5 μg of total protein being applied per each lane.

5.3 Translational approach, in vivo x-ray

Two in vivo experiments were carried out to evaluate the efficacy of the drugging regimen in the highly metastatic 4T1 model of breast cancer, which is highly resistant against basically all therapy measures[225]. The few success stories were bought with arduous toxicity[225,259], including temporary weight loss of more than 20 %[225]. In the first experiment, many different treatment groups were evaluated. In the second experiment, the focus was put on XL-184, which revealed the most pronounced effect.

5.3.1 In vivo Experiment I

Dosing was 10 mg per kg body weight for XL184 and Chloroquine and 50 mg per kg body weight for CDDO-Me. Drugging was started at day 10 after tumor inoculation, irradiation with a dose of 10 Gy from one side took place on day 14. The reduction of tumor growth was most pronounced in the group treated with XL-184, with no evident influence of the radiation at day 19 (figure 16). Interestingly, the combination of the three drugs did not lead to an additive effect, which somehow reflects the results in the in vitro setting. For CDDO-Me alone, the tumor growth curve was practically identical with the control group (data not shown). Instead of an effect on the tumor growth, the mice receiving any CDDO-Me containing diet responded with weight loss of about 10 % (data not shown). The irradiated groups receiving control diet and CDDO-Me treated diet were lost shortly after irradiation demonstrating severe symptoms of illness and dramatic weight loss, which led to termination of the experiment at day 19. A difference in lung metastasis count was not seen under these conditions (data not shown).
In the other treatment groups, the effect on tumor size was between the control group and the groups treated with XL-184, pointing to no additive or otherwise increased effect (figure 17).

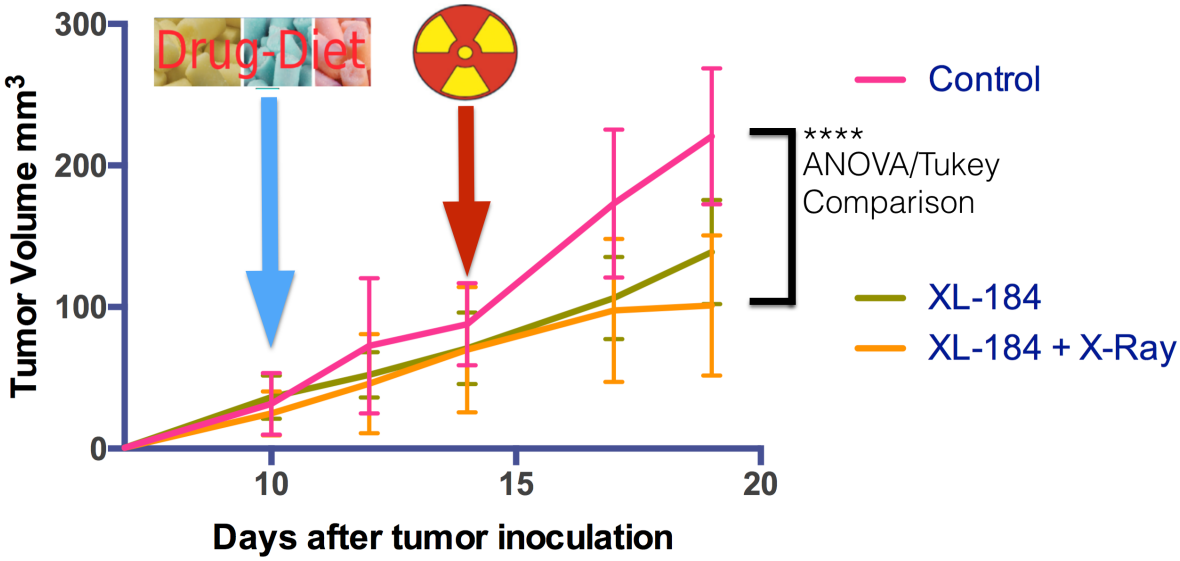

Figure 16: Tumor growth kinetics of 4T1 tumors under influence of drugging with XL-184 starting at day 10 and irradiation with 10 Gy x-ray at day 14. Each group did consist of 8 animals.

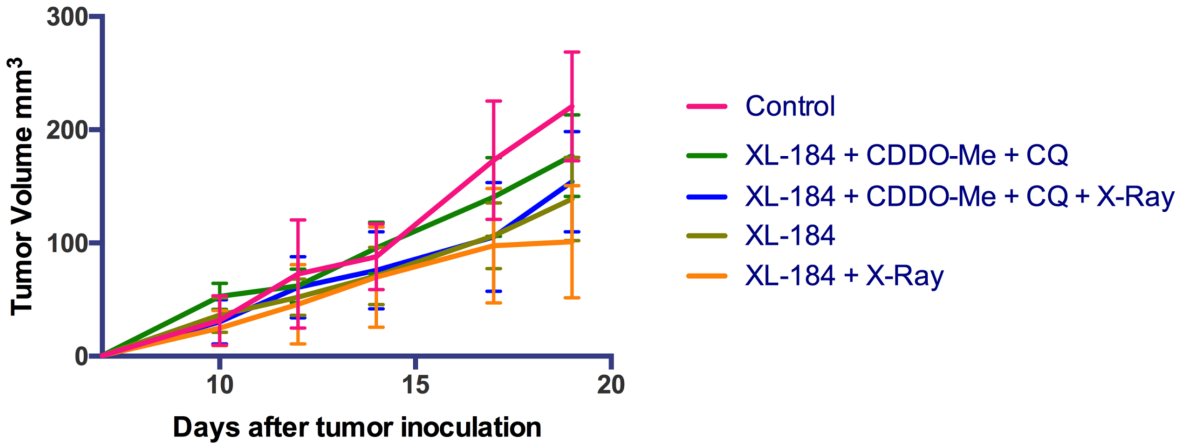

Figure 17: Tumor growth kinetics of 4T1 tumors under influence of drugging with XL-184, CDDO-Me and Chloroquine starting at day 10 and irradiation with 10 Gy at day 14. Animal work was carried out under the responsibility of Jutta Petschenka by TRON GmbH.

5.3.2 In vivo Experiment II

In the second experiment, the beginning of the drug treatment was started at day 5 after tumor inoculation. The radiation dose was increased to 14 Gy. Irradiation also took place on day 14. The number of treatment groups was narrowed down to the control groups and the treatments which included XL-184. The treatment groups 1 and 2 did consist of 13 animals, the other groups of 8 animals per each group (figure 18).

All treatment groups showed slower tumor growth than the control group (figure 18). The

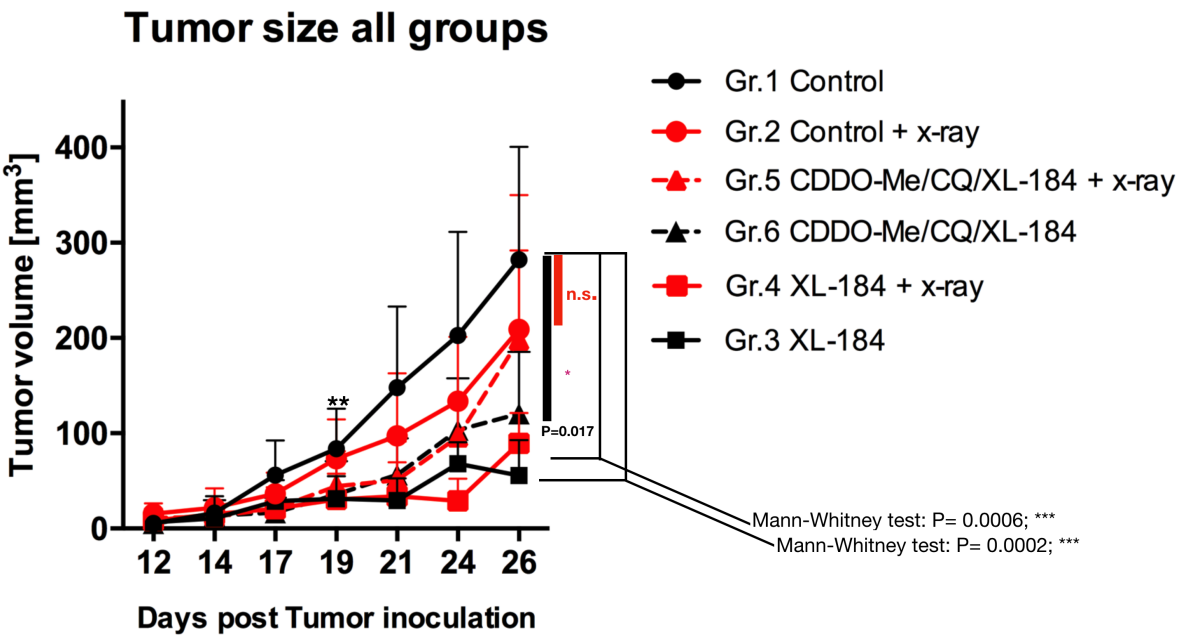

Figure 18: Tumor growth kinetics of 4T1 tumors under influence of drugging with XL-184, CDDO-Me and Chloroquine starting at day 5 and irradiation with a dose of 14 Gy x-ray at day 14 after tumor inoculation. Animal work was carried out under the responsibility of Jutta Petschenka by TRON GmbH.

irradiated group (14 Gy on day 14) showed only a small difference to the control group till day 19, with the difference getting more pronounced at day 21. The strongest tumor growth reduction was found in the group treated only with XL-184. In the group where XL184 and irradiation was combined, irradiation did not lead to further tumor growth reduction in comparison to the group treated with XL184 alone. Treatment of the tumors with all three drugs and irradiation revealed a tumor growth reduction compared to the control group. However, the effect was inferior to the growth reduction with XL-184 alone.

At day 19, the groups revealed a two star significance in the ANOVA test for difference among the means of the groups (P = 0.0038). For the groups treated with XL184 and XL184 + 14 Gy, at day 19 the Mann-Whithney - test revealed significance with a P of 0.0056 and 0.0063, respectively, concerning the difference to the control group. The difference of these two treatment groups to the control groups increased at day 26, with a statistical significance of P = 0.0002 and P = 0.0006, respectively. The Mann-Whitney test was resulting in significance (P = 0.017) in the group which was not irradiated but treated with all drugs in comparison to the control group. At day 26, the difference of the irradiated group to the control group was not statistically significant (figure 18).

Upon resection of tumors and organs, the lungs of all animals were infused with blue ink and the now visible superficial metastases were counted without microscopic aid. Also with the now extended observation time frame compered to the first *in vivo* experiment, no relevant difference in lung metastases count was seen (figure 19).

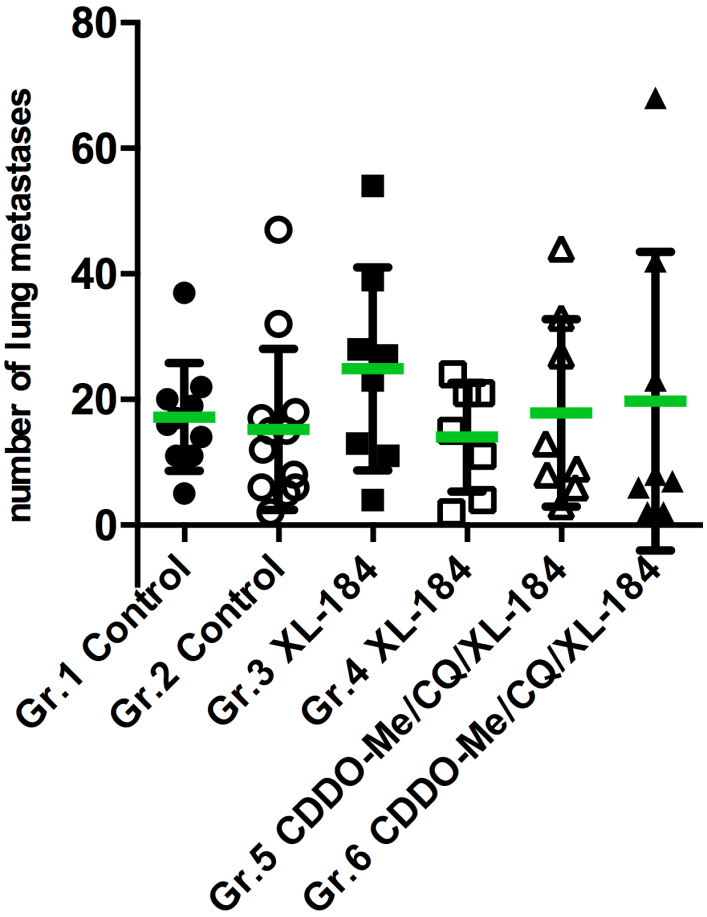

Figure 19: Superficial lung metastases were counted macroscopically in all animals of each group after sacrifice on day 26 after tumor inoculation. Each data point represents the number of counted metastases in one animal. Groups 2, 4 and 5 were irradiated with 14 Gy of x-ray irradiation from a 320 kV x-ray tube.

6 Discussion

6.1 Phenotype changes of tumor and immune cells upon irradiation

6.1.1 Exposure of MHC-I and CD95

The comparison of x-ray and carbon ion irradiation revealed a somewhat inferior ability of carbon ions to induce surface translocation of MHC-I and CD95 in CT26.WT cells (figures 4 and 5). From a perspective of immune surveillance, this might be seen as a disadvantage, as less MHC-I on tumor cells will not improve tumor antigen recognition[121]. In the preliminary measurements with 4T1 cells, a difference in the generally low surface translocation between x-ray and carbon irradiation was not visible (figure 6). However, the exploration of immune relevant molecules in the tumor microenvironment after irradiation has only begun[214,310], and huge differences between *in vivo* and *in vitro* results were seen for surface translocation of immune relevant molecules[311]. So, the difference between *in vitro* work and *in vivo* reality can by far exceed the difference between radiation qualities. Therefore, a deeper exploration of radiation effects on tumor cells and their surrounding stroma in immune competent and -incompetent models side by side could be a way to increase the insights.

6.1.2 Induction of Autophagy

Mechanistically, it is interesting that pharmacological inhibition of mTor e.g. with rapamycin, which usually leads to the induction of autophagy, has been shown to decrease MHC-I surface translocation[312], while irradiation[312] including carbon ion irradiation is increasing it. However, upon carbon ion irradiation, no excessive amount of LC3-RFP - spots indicating autophagy were spottet in U2OS cells at doses up to 30 Gy after 24 h in one experiment. Contrariwise to the results with carbon ion irradiation, there was a response 24 h after 10 Gy of x-ray (figure 7). On the other hand, there are reports of autophagy induction via carbon ion irradiation in other tumor cell lines[313,314]. For my experiments, only one cell line was at hand, so a systematic exploration of dose and radiation quality dependence in autophagy induction and exploration of mechanisms in more models could be useful.

6.2 Characterization of Molecular Mechanisms

The apparently lower propensity of carbon ions to induce an enhancement of MHC-I surface presence on tumor cells (figures 1 and 2) suggests, that an increase by pharmacological means could be a useful way to complement carbon ion therapy - apparently even more than in the case of classical photon therapy. Therefore, more knowledge on the molecular mechanism of surface translocation is needed. To approach this challenge, the CT26.WT colorectal carcinoma cell line, which is triploid in double mutated K-Ras (V8M, G12D, see figure 1)[303], was treated with a set of highly selective inhibitors of pathways perpetually active in this cell line

(see also figure 1). Preliminary results showed an increased MHC-I surface translocation after selective MEK - Inhibition (data not shown).

6.2.1 Switch from mesenchymal to epithelial phenotype via MEK - Inhibition

The experiments with the MEK inhibitor trametinib revealed an interesting phenotype change from the spindle shaped, mesenchymal CT26.WT cells[315] to a flat, epithelial phenotype (figure 11). Inhibition of MEK[307] and Erk1/2[308] was shown in other cell types to reverse epithelial to mesenchymal transition (EMT)[118,165], though not in this readily visible manner. EMT is a radiation inducible[174] signaling program involved in invasive growth, and therefore cancer metastasis[316]. Inhibition of Erk1/2 (downstream of MEK, see figure 1) was shown to reverse invasive growth[317], in line with this visible phenotype change. Because of its involvement in disease progression, treatment resistance and worsened prognosis, pathways involved in EMT are now associated to the cancer stem cell[316,318,319], which is considered a 'cornerstone of radioresistance'[320]. Adding to that, EMT is now increasingly recognized as a resistance mechanism to immune therapy[166,321,322], and also relevant for radiotherapy[174].

6.2.2 Apoptosis induction via X-Ray and combined Inhibition of MEK and Met

Targeting treatment resistance pathways to enhance radiotherapy efficacy is an upcoming challenge. Inhibition of MEK was shown to sensitize K-RAS mutant cell lines for radiation[323], and is here combined with selective inhibition of c-Met. C-Met is a radiation-[324] and hypoxia[268] inducible drug target involved in EMT, CSC properties and radioresistance[320]. Therefore, inhibition of c-Met is also seen as a tool for sensitizing c-Met positive cancers to radiation[63]. Irradiation of drugged and undrugged cells and combined treatment with the inhibitors did show a modest increase in apoptotic cells after 24 h in this preliminary experiment (figure 12). K-RAS mutations were reported to confer resistance to Met Inhibition[325], which was apparently not vastly improved targeting MEK in this relatively strongly mutated cell line as predicted by the transcriptome[303]. Given the inherent heterogeneity of *evolving*[35] tumors[326] and the tumor microenvironment[248], it is foreseeable that single target selective inhibitors will meet limitations. Additionally, conditions like hypoxia and radiation could induce even more cancer promoting pathways, leaving loopholes for tumor recurrence. The role of apoptosis induction is not restricted to direct effects of drugs and / or radiation,but also seen as an immune relevant process, as apoptotic cells were shown to be more likely to activate dendritic cells, enhancing their cross presenting activities[146,182]. This does apparently not apply to necrotic cells in the same way[161,327]. This means that more efficient induction of apoptosis is desirable, which probably could be furnished more straightforwardly by agents with multiple drug targets, which also strike more or less generally abundant features of the tumor microenvironment (see figure 4).

6.3 Translational approach, in vitro

Three drugs chosen mainly for their reported immune modulatory properties (see introduction) were characterized for their direct effects on different murine tumor cells, regarding their influence on apoptosis. Evaluation on possible toxic effects was carried out using neonatal human microvascular endothelial cells (HMVEC), representing normal tissue.

6.3.1 Induction of Cell Death in murine CT26.WT, B16-F10 and 4T1 tumor cells

In CT26.WT colorectal carcinoma cells and B16-F10 melanoma cells, the combination of plasma typic doses of chloroquine, CDDO-Me and XL-184 (cabozantinib) did reveal an increase in dead tumor cells which was more pronounced in combination with x-ray irradiation (figures 13 and 15), and even further increased with heavy ions (figure 14). This was also the case in combination with carbon ion irradiation in experiments with CT26.WT cells, which were the focus cell line in the in vitro experiments, also because of the comprehensive characterization of this cell line[303]. In the B16-F10 murine melanoma cell line[304], this efficacy could potentially be ascribed to high levels of c-Met and Axl as seen in the transcriptome, rendering the cells susceptible to XL-184, which is targeting Met, Axl, and all the other receptor tyrosine kinases depicted in figure 1.

In the 4T1 cell line, the behavior was different. XL-184 alone did show cell killing capabilities superior to the triple combination under irradiation conditions. Additionally, on 4T1 cells, there was nearly no Annexin-V detectable under any evaluated experimental conditions with and without x-ray irradiation (see figure 16). This is resembling the reported reluctance of 4T1 cells to undergo apoptosis after irradiation, tested with caspase inhibitors, which did not influence irradiation induced cell death[328]. p53 is amongst the most frequently mutated tumor suppressor gene in about half of all cancer cases[283], and nearly all of ovarian cancers[3]. In B16-F10 cells, p53 is present, but mutated in the DNA binding region[304]. The B16-F10 melanoma cells did show a pronounced apoptotic response to radiation with chloroquine, which was reported to support p53 mediated apoptosis in glioma cells[329]. In melanoma, chloroquine activity was also ascribed to autophagy inhibition[330] and lysosomal membrane destabilization[331]. Radiosensitization by chloroquine was reported back in 1973[332].
CT26.WT, which responded with apoptosis, is expressing wild type p53[303]. Unlike the 4T1 cell line - in which p53 protein is generally absent[333]. This result is reflecting the notion that the plethora of observed p53 mutations does not necessarily hamper tumor cell apoptosis[3], as apoptosis (at least, Annexin-V surface translocation) was seen in B16-F10. But it also lends one more explanation for the excruciating resistance of the 4T1 model to radiotherapy[259], immune therapy[283] and chemotherapy, given the role of p53 in the induction of cell intrinsic apoptosis[56] and cell cycle arrest (see also figure 1). Consequently, the combination of the three pharmacological entities did show in vitro efficacy in apoptosis competent tumor cells.

6.3.2 No Induction of Apoptosis in HMVEC normal tissue cells

Treatment limiting toxicity is a topic of concern also in combined treatments, and in the case of Sunitinib directed against VEGFR2 (and c-Kit and others), dose reductions were made in conjunction with stereotactic body radiation therapy[179], where high, ablative doses are applied to the tumor[153]. Therefore, it could be useful to apply therapy measures directed against tumor cells to physiologically normal cells, to get at least a rough idea about normal tissue toxicity. Drugging HMVEC with a cocktail of Chloroquine [5 μM], CDDO-Me [150 nM] and XL-184 [2,5 μM] did not result in a lot of dead cells, at least 95 % of cells were surviving with and without radiation and / or drugging (figure 17). A clinical dose reduction trial was untertaken with XL-184 in colorectal cancer without radiation, and did show sustained treatment efficacy despite the dose reduction to 40 mg per day[334]. This is pointing to a possibility to augment radiotherapy in a tolerable manner. Drug induced toxicities are very prevalent also with targeted agents[335] and combined toxic effects of targeted therapies with x-ray can e.g. severely affect the skin[336]. Therefore, carbon ion therapy with its precise dose applicability and lower skin dose could be superior in preventing toxicities of combined treatments. More test systems for such questions could be useful.

6.3.3 Western Blot Analysis of anti-apoptotic molecules, p-Akt, p-Erk & p-STAT3

As CT26.WT is highly expressing wild type p53[303], the question could be raised why it is difficult to drive CT26.WT cells into apoptosis *via* irradiation, where it overall rather acts *via* necroptosis, which is immunologically less favorable[146,161,182,327]. The transcriptome of CT26.WT[303] hinted to an overexpression of the antiapoptotic molecules indicated in figure 1 and the tyrosine kinases Met and Axl (see figure 1). As we could see apoptosis under the drugging regimen, western blot analysis was undertaken to characterize the protein levels of Bcl-XL, Mcl-1, and Survivin and the phosphorylation states of p-Akt, p-STAT3 and p-Erk1/2 (see results on figure 18), which all are considered to contribute to disease progression and therapy resistance (see figure 1 and figure 2). The drugging regimen was as above consisting of chloroquine [5 μM], CDDO-Me [150 nM] and XL-184 [3,3 μM] and was applied for 44 h to CT26.WT cells. The western blot analysis, carried out at least in triplicates but only from one experiment, revealed a reduction of the protein levels of BCl-XL, Mcl-1, and Survivin which was attributable mainly to CDDO-Me, as well as a reduction in phosphorylated Akt and STAT3. Reduced levels of phosphorylated Erk1/2 were also seen under the influence of XL-184, in line with previous reports of the effects of XL-184 on neuroblastoma[337] and the prediction from the cell free assay offered by Uitdehaag et al.[257] (see also figure 4 for more targets and their function). This hints to the mechanisms by which the drug combination could drive the CT26.WT cells into apoptosis.

6.4 Translational approach, in vivo

The question how to best augment particle therapy can only be answered by including the tumor (micro)environment and the immune system[45,338]. The most difficult, highly treatment resistant diseases are prime targets for heavy ion cancer therapy[339]. The 4T1 model in BALB/c mice we used here is known to be p53 negative[333], does not respond to irradiation with apoptosis[328] and did not appear to undergo apoptosis in our experiments (see above). It can not be cured with 60 Gy of x-ray irradiation[259], has few mutations, is therefore less immunogenic[11] and kills animals with lung metastases in 4 weeks. This is reflecting a tough situation in the clinic, and therefore the 4T1 model is already appreciated by the heavy ion community[339].
The drugging with Chloroquine, XL-184 and CDDO-Me per animal chow at low doses (see methods) was applied to 4T1 tumor bearing mice starting at day 10 in the first experiment and at day 5 in the second experiment with groups narrowed down to the diets containing XL-184 (figures 17 and 18). Key results in brief:

- **Markedly reduced tumor growth** in the XL-184 treated groups.
- **No apparent reduction in lung metastases** in most animals, some animals potentially did respond, but no overall change.

6.4.1 Interpretation of the results

Tumor growth
Without drugs, x-ray irradiation did lead to some minor tumor growth retardation, in line with the results of Verbrugge et al.[328] (figure 19). XL-184 markedly reduced tumor growth despite the low dose of 10 mg/kg per mouse and day (figure 19). In the transcriptome of 4T1, it can be seen that two actionable drug targets of XL-184, the tyrosine kinases Axl and Met, are overexpressed comparable to CT26.WT cells[303]. XL-184 can inhibit all Receptor Tyrosine Kinases shown in figure 1 and the tyrosine kinases Src and Erk (figure 1)[257]. While Met is long known for its notorious role in cancer (https://resources.vai.org/Met/Index.aspx), Axl gained popularity more recently (see figure 1) e.g. in conferring resistance to targeted drugs[62,340,341]. Other XL-184 drug targets[257] are expressed to a lower extent in 4T1: Brk1, Ddr1, Lck, Lyn, PDGFR-α, Tnk1 and Tyro3. Drug targets expressed in 4T1 as seen in the transcriptome but not targeted by XL-184 are FGFR and Her-2[342], pointing to further optimizations. Histological analyses like staining for phosphorylation states of molecules shown in figure 1 and screen cell types present in the tumor will help to see if this growth suppression was based on direct effects on tumor cells, immune mediated or maybe both. The extent of growth reduction enabled by XL-184 seen here was comparable to the recent results of Wolchok et al. with 4T1 mice treated with dual checkpoint (CTLA-4, PD-1) and PI3Kγ

inhibition[120].

Remarkably, in the drugged mice, the tumors of the mice which also received irradiation were apparently growing slightly faster toward the end of the experiment (figure 19). Even though this difference was small and non significant, it probably should be not completely ignored, as it is reminding of the results of Cui et al., who reported increased phosphorylation of Akt and STAT3 and EMT after x-ray irradiation[135]. It can not be completely excluded that x-ray irradiation was partly overriding the pharmacological intervention (see figure 1 for a possible mechanism).

Lung Metastases

The immune modulatory properties reported for chloroquine, CDDO-Me and XL-184 (see introduction) were not sufficient to reduce lung metastases count in combination with and without irradiation (figure 23). Lung metastasis in the 4T1 model is clearly dependent on myeloid cells[120,221,225]. The drug combination was also directed against myeloid cells[263,287] (see introduction, figures 1 and 3). The activity of the drugging regimen against Akt[170] and Erk1/2[122] (see figure 18) was thought to reduce levels of G-CSF, and consequently, levels of the myeloid cells in question[122,170,255] (see figure 1 [Erk1/2 -> G-CSF -> MDSC]). Considering the effect of radiation on tumor growth kinetics under drugging conditions (figure 19), this lack of efficacy could eventually be attributed to the influence of x-ray irradiation on myeloid cell levels, and it is acknowledged to be (one of many) crucial open challenges awaiting pharmacological solutions[343]. A closer examination of the serum samples on growth factors and cytokines including G-CSF will probably reveal more insights.

6.5 The Particle Perspective

A key question is of course, if carbon ion irradiation would be more efficient in local control and metastasis reduction by immunological means[344]. Given the results concerning tumor growth, lung metastasis and the role G-CSF - induction, it is interesting how carbon ion irradiation would influence the phosphorylation states of Akt[170]. In preliminary experiments, we found no increase in Akt and Erk phosphorylation after carbon ion irradiation, a finding also encountered by other researchers[314,345,346]. Given Therefore, it can be estimated that carbon ion irradiation would demonstrate a more beneficial behavior in the 4T1 model, in line with the immune activating properties of particle irradiation[145,344,347]. Irradiation of human skin was recently shown to have immune suppressive properties[348,349]. However, as the depth dose profile of carbon ion irradiation is inversed compared to x-ray[350], resulting in

much smaller skin doses (and fewer or no skin reddening at all), the immunological effects of particle irradiation could even be superior to x-ray.

The radiotherapy field in general is now undertaking massive efforts toward combined treatments[256] including immunotherapy[153,226]. The immunotherapy field on his behalf is adopting methods to improve immunotherapy with small molecules[52,120] and radiation[351]. The small molecule drug XL-184 alone was at least as effective as nivolumab (anti PD-1) for patients with poor prognosis[352]. Combining the versatile features of different therapy measures in the frame of determined, structured and sustained efforts, will enable to vastly improve patient benefit from particle therapy.

7 Methods

7.1 Cell Culture

CT26.WT, 4T1 and B16-F10 cells were purchased from ATCC. CT26.WT and 4T1 cells were expanded in RPMI (Millipore), supplemented with 9,6% FBS (Biochrom) and +1% Penicillin/Streptomycin to yield a cryostock of cells used after few passages for the experiments. B16-F10 cells were cultivated in DMEM (Biochrom) also supplemented with 9,6% FBS (Biochrom) and +1% Penicillin/Streptomycin. Cells were passaged shortly before reaching confluence.

7.2 Drug treatment of Cells

Cells were incubated for 20 h with Medium supplemented with Drugs in DMSO or Water typically using a 1:1000 to 1:2000 dilution and irradiated with 15 Gy. Chloroquine was purchased from Sigma and dissolved in PBS to yield a 10 mM Solution, XL184 Malate and CDDO-Me were purchased from Selleckchem, diluted to 10 mM and 300 μM in DMSO, respectively and stored at -80 C. RPMI supplemented with 10 % Fetal Calf Serum and Penicillin/Streptomycin was admixed with stock solutions of drugs at the appropriate ratios (1:3000 to 1:1000) to yield the indicated concentrations.

7.3 Flow Cytometry

Cells were removed from the culture vessels and petri dishes with Accutase after washing with PBS, keeping the supernatant, eventually containing apoptotic cells. After Accutase incubation, the dissociated cells were collected using the supernatant from the specific probe using no pipetting but just careful swapping of liquids to keep shear stress as absent as possible. Staining was carried out carefully with fewer than 1 Million Cells with antibodies diluted in Staining Buffer for the respective molecules.

7.3.1 Staining for Apoptosis and Necrosis

Staining with DAPI and Annexin-V is a standard method for the characterization of necrosis and apoptosis. The blue fluorescence of the DNA binding dye DAPI can be measured after excitation with light in the violet (405 nm) part of the spectrum, but only in cells with a membrane which is permeabilized by cell membrane damage, which is the hallmark of necrosis. DAPI is not able to penetrate intact cell membranes. Therefore, viable cells are DAPI negative. Upon apoptosis, phosphatidylserine, which is prevalent on the inner part of the membrane, is turned to the outer leaflet on the membrane. Annexin-V has a high binding affinity to phosphatidylserine, by means of the coordination of calcium with phosphate residues. The staining buffer therefore is prepared to contain some amount of Ca^{2+}. Therefore, the staining

Buffer was prepared supplementing 1 liter Millipore Water with 10 ml 1 M HEPES, 8,18 g NaCl and 368 mg $CaCl_2$ * 2 H_2O. It has been observed that apoptotic cells also have a tendency to non specific binding (data not shown). Therefore, to evaluate surface molecules like MHC-I (H2-kD), a gating strategy was devised to select cells which were both DAPI and Annexin-V negative. In earlier experiments, instead of DAPI, Propidium Iodide was used, mainly with the CT26.WT cells, which has a flourescence emission in the red and infrared part of the spectrum. The FACS gating strategy was inclining to prefer DAPI, in order to leave the red channel to the evaluation of surface molecules. Additionally, the stainings with DAPI had a somehow cleaner appearance with more distinct populations of living versus dead cells.

7.3.2 Staining for surface molecules

Antibodies used were: MHC-I (H2-kD, clone SF1-1.1) (BD Bioscience, Heidelberg), and CD95/Fas (eBioscience, Clone Jo2) and Annexin-V conjugated to FITC (eBioscience). After centrifugation at 300 g, cells were resuspended in Staining Buffer containing 1 μg DAPI and secondary antibodies donkey against rat conjugated to APC, goat anti rabbit conjugated to Alexa Fluor 488 or APC-Cy7 (Santa Cruz, Heidelberg Germany) depending on the respective experiment. Preferably, directly conjugated antibodies were used in later experiments, as this is allowing to spare an additional washing step, further reducing overall shear stress. Nonetheless, in FACS-Histograms, after effective radiation doses and drugging, a lot of smaller particles can be found, likely consisting of cell fragments from apoptotic cells, thus potentially evading analysis. After staining, the cells were subjected to flow cytometric measurements using a FACSCanto II (BD Bioscience).

7.4 Western Blotting

Snap frozen cell samples (LN) were lysed in RIPA Buffer. This solution was supplemented with 1 mM PMSF (Sigma) from a 250x Solution in 2-propanol, and additional Protease / Phosphatase Inhibitor from Pierce (with EDTA). Protein concentration was determined via Bradford Test in duplicates from triplicates using the Roti-Quant - Solution from ROTH, Karlsruhe according to the indicated protocol, with the samples diluted 1:200 in the RIPA Buffer without PMSF. As Protein Standard served BSA from Pierce. The protein standard was diluted from the samples in the range of 5, 10, 20 and 40 μg/ml to serve as standard solutions for the bradford test. The Proteins were separated on Any kD Gels with 10 wells from BioRad, Bio-Rad Laboratories GmbH Heidemannstrasse 164 (Order No. 4569033), after dilution in SDS PAGE sample buffer containing 20% mercaptoethanol. The gels loaded with 7.5 to 20 μg protein were subjected to Gel Electrophoresis at 400 mA using the MiniProtean tank System from BioRad, using Polyvinylidenedifluoride (PVDF) membranes from Merck, Darmstadt. The membranes were blocked in 2 % milk powder in TBST for at least two hours,

followed by incubation with antibodies from Cellsignaling, Schuttersveld 2, 2316 ZA Leiden, Netherlands and secondary anti - rabbit antibodies from Sigma-Aldrich (Sigma-Aldrich Chemie, Taufkirchen) at a concentration of 1:10 000. The resulting blots were developed with the ECL system from thermo fisher. The ECL-treated membranes were quantified using a camera system Fusion FX7 from Vilber Lourmat, Eberhardzell. The used antibodies and their respective dilutions in 2 % Milk powder in TBST buffer were the following:

Antibodies for evaluation of phosphorylation states were from Cellsignaling:
Phospho-Akt (Ser473) (D9E) 4060: 1:1000;
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) 4370: 1:2000;
Phospho-Stat3 (Tyr705) (D3A7): 1:500.

Antibodies for evaluation of the levels of anti - apoptotic molecules were from Cellsignaling:
Bcl-xL (54H6) mAb 2764: 1:2000;
Mcl-1 (D35A5) mAb 5453: 1:500;
Survivin (71G4B7) mAb 2808: 1:500.

The antibody for the housekeeping gene vinculin was from santa cruz: sc-5573: 1:250

All primary antibodies used in western blot listed above were produced in rabbit.

7.5 x-ray irradiation of cells

Cell samples were irradiated with an 250 kV X-Ray tube from General Electrics and a cathode current of 16 mA. Low energy X-Rays were filtered with 7 mm Be, 1 mm Al and 1 mm Cu. The applied dose was measured with a SN4 Dosimeter (PTW Freiburg) using an ionization chamber placed directly under the sample. The dose rates used were between 1.5 and 3 Gy/min.

7.6 Carbon Ion Irradiation

Irradiation with carbon ions was done with the UNILAC linear accelerator furnishing carbon ions with 6.5 MeV/u, the energy on target was 4.0 MeV/u with a LET in H_2O of 325 keV/mum for the data presented in figure 5. The energy of the particles presented in figure 11 was 11.4 MeV/u, the energy on target was 9.9 MeV/u with a LET in H_2O of 168 keV/μm. Two days before irradiation, the cells were seeded in petry dishes from Nunc on a surface of 35 cm^2 at a density of about 80 000 cells per vessel. The radiation doses applied with the UNILAC were calculated as usual[353–355].

7.7 Drugging of Animals

All animal experiments were carried out by the center for translational oncology, TRON, in Mainz. Animals were drugged with a purified ingredient diet prepared by Researchdiets Inc. (New Brunswick, USA) containing 66.6 mg/kg Cabozantinib malate salt - an approach used successfully by James Hodge et al. using the MC38-CEA model in B57/BL6 mice[263]. Cabozantinib malate salt was purchased from LC-Labs (Woburn, USA). 66.6 mg/kg Chloroquine diphosphate from Aldrich and 50 mg/kg CDDO-Me from Selleckchem (Distributor: Absource Diagnostics GmbH, Munich), alone and combined. Glyceryl trioctanoate 37.5 ml and 10 ml Ethanol per kg of diet were used as an adjuvant to dissolve CDDO-Me to ensure appropriate mixing with the diet compounds according to the advice / recipe of the inventor, Dr. Sporn (personal communication). Each diet was mixed with traces of stains in different colors to ensure appropriate feeding during the experiment. After mixing, the diet was pelleted to prevent access to air and possible compound degradation, with the heat produced upon pelleting evaporizing the added ethanol. The product was packed in aluminum coated resealable foil bags at 500 g per bag. The bags were vapor sterilized with H_2O_2 before entering the barrier facility. However, the animals were behaving differently encountering the diet with some animals responding well and some rather inferior upon diet change (apparently discernible to groups of mice in different cages). Additionally, some mild weight loss of 5-10 % was encountered in animals receiving CDDO-Me- but not XL-184 treated diet compound, contrariwise to any publication in the field in at least five mouse models[285]. This could be a peculiarity of the BALB/c strain. The dosing used for XL-184 and Chloroquine, resulting in about 10 mg/kg per mouse per day is in the lower range of concentrations used, with other experimentators using as much as 3-10fold doses, hence representing a low dose drugging[239,356].

7.8 Irradiation of animals

Animals were irradiated using a 320 kV X-Ray tube from X-Strahl equipped with a thoraeus filter consisting of 1.5mm Al, 0.25mm Cu and 0.75mm Sn. The irradiation was carried out with irradiation from above using a beam collimation system. Mice were kept under anesthesia during irradiation, non - irradiated mice were anesthetized equally.

7.9 Metastases count

After sacrifice of the mice *via* cervical dislocation, mice were resected, the trachea were prepared removing skin, muscle and glandular tissue. Trachea were injected with 300 μl of blue ink, the injection site closed with forceps and the lung quickly removed. The lungs were placed in 4 ml Fekete solution at 4 degrees celsius over night. Visible metastases were counted (see figure 20) and the lungs transferred to GSI in histofix (information obtained from TRON

GmbH).

Figure 20: Image of a lung to illustrate the method used to count lung metastases. Image Courtesy: TRON GmbH, Mainz

7.10 Transcriptome analysis

For transcriptome analysis, a mouse reference genome (mm9) was indexed and the RNA - sequence data of the transcriptomes of 4T1, B16-F10 and CT26.WT was aligned with bowtie. The resulting data was transformed to a table using SAMMate. The transcriptome data used here is available at http://sra.dbcls.jp.

7.11 Statistical analysis

Graphpad Prism for Apple from GraphPad Software, La Jolla California USA, www.graphpad.com was used. For statistical analysis see Figures 4,5,6 (error bars represent the standard deviation of three measurements from one representative experiment). For figures 16, and 18 statistical analysis is based on two independent experiments with at least 8 animals per group.

8 List of Abbreviations

PBS for cell culture was used as supplied from Biochrom.

RPMI for cell culture was used as supplied from Biochrom.

ATP Adenosine triphosphate

ATCC American Type Culture Collection

Axl Tyrosine-protein kinase receptor UFO

B16-F10 Murine melanoma cell line capable of lung metastasis (slower than 4T1).

Bcl-2 B-cell lymphoma 2

BCl-XL B-cell lymphoma-extra large

Bim Bcl-2-like protein 11 (pro apoptotic)

Brk1 Probable protein BRICK1

Btk Bruton's tyrosine kinase

CAR-T-Cells Chimeric antigen receptor T cells

CC Cell cycle

CCL2 Chemokine (C-C motif) ligand 2

c-Met Hepatocyte growth factor receptor (HGFR), also known as the c-Met oncogene

CD Cluster of Differentiation / Cluster of Designation / Classification determinant

CDDO-Me from Selleckchem was diluted in DMSO from Roth for stock solutions for cell experiments and forwarded as received to researchdiets for *in vivo* experiments.

Ddr1 Discoidin domain receptor family, member 1

CSC Cancer stem (like) cells

CT26.WT Murine colon cancer cell line, chemically induced.

CTLA-4 Cytotoxic T-lymphocyte-associated protein 4 (CD152)

DAPI 4',6-Diamidino-2-phenylindole

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DR5 Death receptor 5

EMT Epithelial Mesenchymal Transition

Erk1/2 Extracellular signal regulated kinases, (subgroup of MAPK)

FACS Fluorescence-activated cell scanning

FAP Fibroblast activation protein

Fas No genuine abbreviation for this "death receptor". Based on the name of an antibody termed "anti Fas" (CD95), before the gene was discovered.

FBS Fetal bovine serum

FGFR Fibroblast growth factor receptors

Flt-3 Fms like tyrosine kinase 3, fetal liver kinase-2, CD135

G-CSF Granulocyte-colony Stimulating Factor

Glyceryl trioctanoate was used as an adjuvant for the preparation of the drugged research diet and was purchased from Sigma in the highest possible quality.

GSH Glutatione

GSK-3 Glycogen synthase kinase 3

Gy Gray (1 Gy = 1 J/kg)

Her-2 Receptor tyrosine-protein kinase erbB-2, human epidermal growth factor receptor 2, (CD340)

HGF Hepatocyte growth factor

HMGB1 High mobility group box 1

HMVEC Human microvascular endothelial cells

IDO Indoleamine 2,3 - dioxygenase

IFN Interferon, a class of key cytokines in antitumor defense

IAP Inhibitor of apoptosis

Kit Stem cell growth factor receptor, CD117, tyrosine-protein kinase Kit

KPC mice K-ras$^{LSL.G12D/+}$; p53$^{R172H/+}$; Pdx^{Cre} mice

LC3 Microtubule-associated proteins 1A/1B light chain 3A

LC3-GFP-RFP LC3 coupled to green flourescent protein and red fluorecent protein

Lck Lymphocyte-specific protein tyrosine kinase

LN Liquid Nitrogen

LPS Lipopolysaccharide

Lyn Tyrosine-protein kinase Lyn, derived from Lck/Yes novel tyrosine kinase (Scr family)

M6PR Cation-dependent mannose-6-phosphate receptor

MAPK Mitogen Activated Protein Kinases

Mcl-1 Induced myeloid leukemia cell differentiation protein Mcl-1

MDSC Myeloid-derived suppressor cell

MEK MAP kinase-kinase, which activates ERK, MAPK/ERK kinase

Mer Proto-oncogene tyrosine-protein kinase

MHC Major histocompatibility complex

MLKL Mixed lineage kinase like

MSC Mesenchymal stromal cells

NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells

Nrf-2 Nuclear factor (erythroid-derived 2)-like

PAGE Polyacryl amide gel electrophoresis

p53 Protein 53, a hallmark tumor suppressor gene

PBS Phosphate buffered saline

PD-1 Programmed cell death protein 1

PDGFR Platelet-derived growth factor receptors

PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase

PTEN Phosphatase and tensin homolog, a tumor suppressor encoded by the PTEN gene.

PUMA p53 upregulated modulator of apoptosis

Raf Rapidly accelerated fibrosarcoma, a kinase family related to retroviral oncogenes

Ras Rat sarcoma (oncogene)

RET tyrosine kinase, abbreviation for "rearranged during transfection"

Ron Recepteur d'Origine Nantais, Macrophage-stimulating protein receptor

ROS Reactive Oxygen Species

RPMI Cell culture medium developed at the Roswell Park Memorial Institute

RTK Receptor Tyrosine Kinase

SBRT Stereotactic beam radiation therapy

SDS Sodium dodecyl sulfate

Src Proto-oncogene tyrosine-protein kinase Src (discovered in the Rous sarcoma virus)

STAT3 Signal transducer and activator of transcription 3

STING Stimulator of Interferon Genes

Tie2 Angiopoietin-1 receptor, CD202B

U2OS Osteosarcoma cell line

UNILAC Universal linear accelerator

TKI Tyrosine Kinase Inhibitor

TME Tumor microenvironment

TNF Tumor necrosis factor

Tnk1 tyrosine kinase, non-receptor, 1

TRAIL Tumor Necrosis Factor Related Apoptosis Inducing Ligand

Tyro3 Tyrosine-protein kinase receptor

VEGF-R Vascular endothelial growth factor receptor

XL-184, also known as Cabozantinib or Cometriq was purchased from Selleckchem as the malate salt and dissolved in DMSO (Roth) for *in vitro* experiments. For *in vivo* experiments, XL-184 was ordered from LC Laboratories, 165 New Boston Street, Woburn, MA 01801 as the malate salt and forwarded to Researchdiets.Inc for preparation of the drugged animal diet.

9 List of Materials

4T1, B16-F10 and CT26.WT cells were purchased from the ATCC and maintained in RPMI (CT26.WT, 4T1) and DMEM (B16-F10).

CDDO-Me from Selleckchem was diluted in DMSO from Roth for stock solutions for cell experiments and forwarded as received to researchdiets for *in vivo* experiments.

DMEM Dulbecco's Modified Eagle's Medium was purchased from Biochrom.

DMSO Dimethyl sulfoxide was purchased from Roth.

FBS Fetal bovine serum was used as supplied from Biochrom to supplement the RPMI and DMEM media.

Glyceryl trioctanoate was used as an adjuvant for the preparation of the drugged research diet and was purchased from Sigma in the highest possible quality.

Glutathione was used as supplied by Sigma.

HMVEC Cells were purchased from Cell Applications, San Diego, CA 92121, USA and maintained in a proprietary CADMEC / HMVEC medium.

KH_2PO4 was supplied by Merck, Darmstadt.

Lipopolysaccharide was purchased from Sigma and prepared as a stock solution in DMSO at one mg per one ml.

Na_2HPO4 was supplied my Merck, Darmstadt.

NaCl was supplied by Roth, Karlsruhe.

PBS for cell culture was used as supplied from Biochrom.

Penicillin / Streptomycin for cell culture was used as supplied by life technologies, Darmstadt.

Phosphate buffered saline was used as supplied by Biochrom for cell culture. For immunohistology, PBS was prepared as a 10x solution from 2 liter purified water, 160 g NaCl, 4 g KCl, 28.8 g Na_2HPO4 and 4.8 g KH_2PO4.

RPMI for cell culture was used as supplied from Biochrom.

RIPA Buffer consisted of 150 mM NaCl, 50 mM TRIS/HCl, 1 mM Na_3VO_4, 1 mM NaF, 10 g/l Nonident P40, 2,5 g/l Deoxycholate, 1 g/l SDS.

SDS PAGE sample buffer was prepared from 0.035 g bromophenol blue, 7 ml glycerol, 6 ml SDS (10 %), 1 ml Tris-HCl (1 M, pH 6.7). 180 μl of this buffer was supplemented with 20 μl of mercaptoethanol to prepare the sample buffer.

TBST buffer 100 ml Tris (1 M, pH 8.0, Sigma), 500 ml NaCl (3M) and 5 ml Tween 20 and diluted with purified water to 1000 ml resulting in a 10x solution.

Water was used as supplied by the water purification device ELGA, Model PF4XXXXM1, using demineralized water from the GSI compound.

XL-184, also known as Cabozantinib or Cometriq was purchased from Selleckchem as the malate salt and dissolved in DMSO (Roth) for *in vitro* experiments. For *in vivo* experiments, XL-184 was ordered from LC Laboratories, 165 New Boston Street, Woburn, MA 01801, USA as the malate salt and forwarded to Research Diets, Inc. 20 Jules Lane, New Brunswick, NJ 08901, USA for preparation of the drugged animal diet.

List of Figures

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10 Declaration according to § 9 Promotionsordnung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe. Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Datum, Ort

Unterschrift